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7. Metabolism of Bradykinin by Peptidases in Health and Disease

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1. Introduction

The enzymatic breakdown of the kinins affects the duration of their biological actions, as the plasma half-life of intravenously injected bradykinin (BK) is in the range of seconds (Erdös, 1966; Kariya *et al.*, 1982; Bhoola *et al.*, 1992). Kinins are cleaved *in vitro* and *in vivo* by enzymes that belong to families such as zinc-metallopeptidases (Skidgel, 1993), serine peptidases and proteases (Erdös, 1966, 1979; Erdös and Yang, 1970; Jackman *et al.*, 1990), astacin-like metallopeptidases (Bond and Beynon, 1995) and catheptic enzymes (Greenbaum and Sherman, 1962). After the discovery by Werle, Rocha e Silva and their associates that kallikrein or trypsin decrease blood pressure by releasing the kinins, bradykinin (BK) and Lys-BK (kallidin), in plasma and that these peptides are then inactivated, the term "kininase" was coined to denote the inactivating enzymes (Frey *et al.*, 1950, 1968; Erdös *et al.*, 1963; Rocha e Silva, 1963; Erdös, 1989; Bhoola *et al.*, 1992; see Chapter 1 of this volume).

Subsequently, a kininase I-like enzyme was shown to release Arg from the C-terminus of BK and Lys-BK (Erdös *et al.*, 1963), whereas kininase II liberates the Phe^8-Arg^9 dipeptide of BK (Erdös and Yang, 1967; Yang and Erdös, 1967). Moreover, kininase II is identical with angiotensin I-converting enzyme (ACE; Yang *et al.*, 1970, 1971; Skidgel and Erdös, 1993). Early studies noted that sulfhydryl compounds protect kinins against enzymatic inactivation (Rocha e Silva, 1963; Erdös, 1966; Frey *et al.*, 1968; Erdös and Yang, 1970) and that Lys-BK can be converted to BK in plasma (Erdös *et al.*, 1963; Webster and Pierce, 1963). BK is also inactivated by removal of Arg^1 by an enzyme in human erythrocytes (Erdös *et al.*, 1963) or in rat lung by aminopeptidase P, both catalyzing the same reaction (Simmons and Orawski, 1992). Vane (1969) has noted the importance of the pulmonary circulation in the metabolism of vasoactive substances such as BK as well as angiotensin I and 5-hydroxytryptamine. These and other pioneering experiments are reviewed elsewhere (Erdös and Yang, 1970; Erdös, 1979; Skidgel and Erdös, 1993).

Lys-BK has nine, and BK eight peptide bonds that can be hydrolyzed (Bhoola *et al.*, 1992). Lys-BK is converted to BK by the cleavage of the Lys^1-Arg^2 bond (Erdös *et al.*, 1963; Webster and Pierce, 1963), and BK subsequently, and possibly simultaneously, is inactivated by other enzymes at any of the eight remaining bonds (Erdös *et al.*, 1963) (Fig. 7.1). Recently, many peptidases that cleave BK have been characterized and their primary structures established (Jackman *et al.*, 1990; Skidgel, 1992, 1993; Bond and Beynon, 1995). Although BK has long been utilized as a convenient substrate for enzyme assays *in vitro*, the importance of several of these mechanisms *in vivo* remains to be determined. Furthermore, although the term "kininase" is convenient to describe an enzyme's action on a single type of substrate, it can be misleading as other substrates (e.g., angiotensin I) may

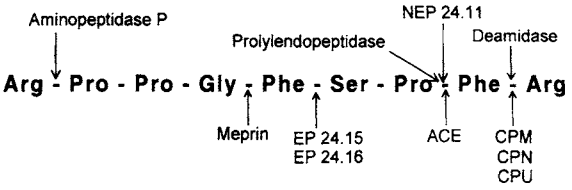

Figure 7.1 Peptide bonds in bradykinin cleaved by peptidases. Arrows show the primary site of cleavage, as established using purified enzymes and bradykinin *in vitro*. NEP 24.11, neutral endopeptidase 24.11; EP 24.15, endopeptidase 24.15; EP 24.16, endopeptidase 24.16; ACE, angiotensin I-converting enzyme; CPM, carboxypeptidase M; CPN, carboxypeptidase N; CPU, carboxypeptidase U.

be cleaved by "kininases". We know now that the kininases cleave many peptides, frequently more avidly than kinins (Skidgel, 1992; Skidgel and Erdös, 1993). Their roles in peptide metabolism depend on their location, access to a particular substrate, and the kinetics of hydrolysis (Skidgel, 1992). A vital question arises in determining which enzymes affect the actions of BK *in vivo*.

With the introduction of therapeutically useful enzyme inhibitors, other questions arise (Gavras *et al.*, 1978; Cushman and Ondetti, 1980; Gavras and Gavras, 1987, 1993; Ondetti, 1994). For example, how much of the effect of a given enzyme inhibitor in the clinic can be attributed to protecting BK, as opposed to other substrates, against enzymatic breakdown? These questions are especially appropriate for ACE inhibitors, which are administered to millions of patients. Inhibitors of neutral endopeptidase 24.11 (NEP or neprilysin, also known as enkephalinase) are also being tested clinically (Elsner *et al.*, 1992). The attractive idea of employing a second generation of inhibitors that can inhibit two enzymes, e.g., both NEP and ACE, will make the issues even more complex (Gros *et al.*, 1991, Gonzalez-Vera *et al.*, 1995, Flynn *et al.*, 1995, French *et al.*, 1994, French *et al.*, 1995). Figure 7.1 illustrates the amino-acid sequence of BK and the sites of actions of peptidases. Of the numerous enzymes that cleave BK, ACE, neutral endopeptidase, aminopeptidase P and carboxypeptidases N and M are probably the most important *in vivo*, although this can vary from species to species.

2. Kininase II (Angiotensin I-converting Enzyme)

Because of the wide distribution of ACE in mammalian species, its presence on the vascular endothelium, and the low K_m of BK with this enzyme (Table 7.1), ACE is a major kininase in most organs. Based upon its physical properties, enzymatic characteristics and immunological

Table 7.1 Kinetic constants for hydrolysis of bradykinin by various peptidases

Enzyme	*Species*	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})	*Reference*
ACE/kininase II	Human	0.18	660	3,667	Jaspard *et al.* (1993)
ACE N-domain	Human	0.54	300	555	Jaspard *et al.* (1993)
ACE C-domain	Human	0.24	480	2,000	Jaspard *et al.* (1993)
Endopeptidase 24.11	Human	120	4,770	39.8	Gafford *et al.* (1983)
Endopeptidase 24.15	Rat	67	2,028	30.3	Orlowski *et al.* (1989)
Meprin	Mouse	520	1,320	2.5	Wolz *et al.* (1991)
Carboxypeptidase M	Human	16	147	9.2	Skidgel *et al.* (1989)
Carboxypeptidase N	Human	19	58	3.1	Skidgel *et al.* (1984b)
Carboxypeptidase U	Human	10,000	7,260	0.7	Tan and Eaton (1995)
Prolylendopeptidase	Pig	7.5	95	12.7	Ward *et al.* (1987)
Aminopeptidase P	Rat	21	720	34.3	Orawski and Simmons (1995)

ACE, angiotensin I-converting enzyme; ACE N-domain, ACE in which the two zinc-binding histidine residues in the C-domain active site (His^{959} and His^{963}) have been mutated to Lys, leaving only the N-domain active site functional; ACE C-domain, ACE in which the two zinc-binding histidine residues in the N-domain active site (His^{361} and His^{365}) have been mutated to Lys, leaving only the C-domain active site functional.

cross-reactivity, ACE enzymes in different species and various tissues are quite similar and likely represent the two-domain somatic ACE (Skidgel and Erdös, 1993). There is also a testicular enzyme, germinal ACE, which contains only the C-terminal half of somatic ACE (Soubrier *et al.*, 1993a,b). From human material, an active, truncated ACE was purified which contains only the N-terminal half (the N-domain) of the somatic ACE (Deddish *et al.*, 1994).

2.1 LOCALIZATION

ACE is present in vascular beds bound to the plasma membrane of endothelial cells as an ectoenzyme, where it cleaves circulating peptides such as BK. It is also present in subendothelial structures, although in lower concentrations, and in epithelial and neuroepithelial cells. Polyclonal antibodies against either the human kidney or lung enzyme could not distinguish ACE prepared from endothelial, epithelial or neuroepithelial cells, or plasma (Skidgel and Erdös, 1993). ACE activity is higher in arterial than in venous endothelial cells in culture (Johnson *et al.*, 1982). The lungs, because of their heavy vascularization, and the capillary beds in the retina and brain (Igic, 1985) are especially rich in ACE. Monoclonal antibodies also located ACE to the human myocardial vascular endothelium (Falkenhahn *et al.*, 1995). Epithelial cells generally contain more ACE than endothelial cells. The human kidney, for example, contains 5–6 times more ACE per unit wet weight than does the lung. In man and most animals, with the exception of the rat, the kidney proximal tubular brush border is a rich source of ACE (Hall *et al.*, 1976; Schulz *et al.*, 1988). ACE activity is also very high in other microvillar structures of brush border epithelial linings, for example, in the small intestine, choroid plexus and placenta (Skidgel *et al.*, 1987a,b; Skidgel and Erdös, 1993).

In the rat brain, high ACE activity was found in the subfornical organ, area postrema, substantia nigra and locus ceruleus. In the human brain, ACE activity is most concentrated in the caudate nucleus (Skidgel *et al.*, 1987a). Possibly the highest concentration of ACE in any tissue was found in the choroid plexus (Igic *et al.*, 1977) on the ventricular surface of the epithelial cells (Rix *et al.*, 1981; Defendini *et al.*, 1983). The choroid plexus may also be the source of ACE detected in the cerebrospinal fluid.

In rat and rabbit brain, ACE was localized by using labeled inhibitors [^{125}I]-MK351A or [^{3}H]-captopril in autoradiography (Rogerson *et al.*, 1995), and the results are in agreement with immunohistochemical studies (Correa *et al.*, 1985; Skidgel *et al.*, 1987a; Sakaguchi *et al.*, 1988; Chai *et al.*, 1991; Skidgel and Erdös, 1993; Rogerson *et al.*, 1995). ACE is also present in many other human and animal tissues including fish gills, the electric organ of *Torpedo marmorata* (Lipke and Olson, 1988), or the housefly (Cornell *et al.*, 1995), and in human male reproductive tract (Erdös *et al.*, 1985), chorionic membranes, and cultured chorion cells (Alhenc-Gelas *et al.*, 1984). ACE also seems to be a well-conserved enzyme. This was confirmed by molecular cloning and sequencing, showing, for example, that the deduced amino-acid sequences of human and mouse ACE are 83% identical (Soubrier *et al.*, 1988, 1993a,b; Bernstein *et al.*, 1989). Even the *Drosophila* ACE has over 60% sequence similarity (Tatei *et al.*, 1995).

2.2 PHYSICAL AND STRUCTURAL PROPERTIES

ACE purified from various human tissues is a single-chain glycoprotein. Its estimated molecular weight ranges from 140 to 170 kDa in SDS-PAGE (Skidgel and Erdös, 1993). The apparent discrepancy in values probably

arises in part from the heterogeneity in carbohydrate content and experimental variability in electrophoretic conditions. ACE is heavily glycosylated, the human renal enzyme containing approximately 25% carbohydrate (Weare *et al.*, 1982). There are differences, however, in the carbohydrate moieties. For example, human renal ACE has only traces of sialic acid, while the human lung enzyme contains up to 20 sialic acid residues per molecule, in agreement with the hypothesis that the lung is the source of circulating ACE (Weare *et al.*, 1982). Owing to its high sialic acid content, ACE is protected from uptake by liver lectins. The distribution of potential glycosylation sites is uneven, the N-domain containing ten and the C-domain seven (Soubrier *et al.*, 1988). This is consistent with the finding of extensive glycosylation (37% by weight) on the naturally occurring active N-domain in human ileal fluid (Deddish *et al.*, 1994). The molecular weight of ACE deduced from the cDNA sequence (without carbohydrate) is 146 kDa (Skidgel and Erdös, 1993).

2.3 ENZYMATIC PROPERTIES

Similar to many other peptidases, ACE is a zinc metalloenzyme. Thus, chelating agents and sulfhydryl compounds inhibit ACE by complexing the active site Zn^{2+} (Erdös and Yang, 1970; Erdös, 1979). ACE has a pH optimum above neutral, and activity drops steeply at acid pH primarily due to the dissociation of Zn^{2+} from histidine in the active center (Ehlers and Riordan, 1990).

Initially, the idea that BK and angiotensin I are hydrolyzed by the same enzyme protein (Yang *et al.*, 1970b, 1971) was controversial as angiotensin I conversion was almost entirely chloride dependent, while cleavage of BK occurs in the absence of Cl^- at approximately 30–35% of the maximal rate (Igic *et al.*, 1973; Erdös, 1979). Studies with rabbit lung ACE showed that anion activation depends on both the structure of the substrate and the pH of the medium (Ehlers and Riordan, 1990). Accordingly, the substrates were divided into three classes, BK being a Class II substrate with a lower activation constant for Cl^- than angiotensin I, a Class I substrate. Weare (1982) described two anion binding sites in ACE, one of them being the primary activation site.

The concentration of Cl^- ions *in vivo* appears to be high enough for ACE to be fully active in most tissues, although, at some sites, Cl^- concentrations may fluctuate enough to regulate ACE activity with some substrates. It is possible that the Cl^- ion sensitivity of the membrane-bound enzyme *in vivo* differs from that of the solubilized, purified enzyme *in vitro* especially with angiotensin I substrate (Igic *et al.*, 1972). Recent studies with membrane-bound recombinant ACE showed that the Cl^-dependence of the N- and C-domains was the same as that determined for the soluble enzyme (Jaspard and Alhenc-Gelas, 1995).

ACE potentially has many functions in tissues other than the vascular endothelium, although these are not as well understood as its role in cleaving circulating or locally released kinins. For example, on the renal proximal tubular brush border, ACE may inactivate kinins that enter the nephron after glomerular filtration, which would otherwise interfere with renal autoregulation (Scicli *et al.*, 1978; Skidgel *et al.*, 1987b; Carretero and Scicli, 1995).

2.4 SOLUBLE ACE

Although the enzyme is bound tightly to plasma membranes, ACE also exists in soluble form. In addition to blood, soluble ACE is found in urine, lung edema fluid, amniotic fluid, seminal plasma, cerebrospinal fluid and in homogenates of prostate and epididymis (Skidgel and Erdös, 1993). The level of ACE in human plasma is very low, around 10^{-9} M (Alhenc-Gelas *et al.*, 1983). Among laboratory animals, guinea-pig plasma has the highest activity ACE (Yang *et al.*, 1971). In humans, plasma ACE activity varies significantly from person to person (Soubrier *et al.*, 1993a).

2.4.1 Release of Bound ACE

ACE is membrane bound with the majority of the protein projecting into the extracellular space. ACE contains a potential 17 amino-acid membrane-spanning region near the C-terminus. A mutant ACE lacking this transmembrane domain was secreted primarily into the medium, in contrast to cells transfected with the full-length cDNA, which synthesized mainly membrane-bound ACE (Wei *et al.*, 1991a,b). Since it is tightly bound to membranes, ACE must be solubilized prior to purification, either with detergent (Erdös and Yang, 1967) or by cleaving it enzymatically from the cell membrane (Nishimura *et al.*, 1976). It was also shown that trypsin-liberated ACE had a lower molecular weight than ACE mobilized from the membrane with detergent (Erdös and Gafford, 1983), providing evidence for the removal of a small anchor peptide by trypsin. The proteolytic release of germinal ACE expressed in cells may involve activation of a protein kinase C (Ehlers *et al.*, 1995). Cleavage sites in the peptide chain for mobilizing the enzyme were established (Beldent *et al.*, 1993; Ramchandran *et al.*, 1994). The so-called α-secretase may be one of the enzymes solubilizing ACE (Oppong and Hooper, 1993). In some lung tissues, a metalloprotease is responsible for the solubilization of membrane-bound ACE (Oppong and Hooper, 1993). The mechanisms underlying release of ACE from membranes *in vivo*, and its subsequent appearance in body fluids, are unclear, but proteolytic cleavage of the membrane anchor peptide on the C-domain is likely. Since the discovery of separate, active single N-domain ACE (Deddish *et al.*, 1994), this issue deserves further scrutiny.

2.5 CLONING AND SEQUENCING

The molecular cloning and sequencing of the cDNA for human (Soubrier *et al.*, 1988) and mouse (Bernstein *et al.*, 1989) enzymes revealed that ACE has two domains, each with a Zn^{2+}-binding site and an active center (Soubrier *et al.*, 1993a). This two-domain ACE was named "somatic ACE", while the enzyme extracted from rabbit testicles and which contains only the C-domain active site was called "germinal ACE" (Ehlers *et al.*, 1989; Kumar *et al.*, 1989; Lattion *et al.*, 1989). Each active site contains two histidines and a glutamic acid to coordinate the zinc atom (Soubrier *et al.*, 1993a), as well as a catalytic glutamic acid, which is presumed to be a base donor. The overall sequence identity of the two domains is 67%, while it is 89% around the active centers (Soubrier *et al.*, 1993a). Although both domains are catalytically active (Wei *et al.*, 1991a), inhibitors react differently with the active sites at the N- and C-domains (Perich *et al.*, 1991, 1994). For example, captopril has a lower K_i for the N-domain active site, while at optimal Cl^- concentrations, the K_i of other inhibitors is lower for the C-domain active site (Wei *et al.*, 1992).

Germinal ACE contains only the C-domain, the C-terminal half of endothelial ACE, and 67 unique amino acids at the N-terminus (Soubrier *et al.*, 1988, 1993a; Ehlers *et al.*, 1989). As is true for the yet unexplained high concentrations of other proteases and peptidases in the genital tract, the function of testicular ACE is unknown. Germinal ACE is probably the most ancient form of the enzyme and a gene-duplication event is likely to have occurred an estimated 600 million years ago to give rise to the two-domain form (Soubrier *et al.*, 1993a,b). Indeed, *Drosophila* ACE is a single-domain enzyme, like testicular ACE, but structurally it represents the soluble form of the enzyme (Cornell *et al.*, 1995).

2.6 VARIATIONS IN ACE ACTIVITY IN DISEASE

After the gene structure of ACE was deduced, a polymorphism was discovered in the gene sequence, due either to the presence or absence of a 287 bp fragment in intron 16. The importance of this insertion/deletion polymorphism (ACE-II/DD) to BK metabolism is not known. In ACE-II homozygotes, ACE activity was lower in T-lymphocytes and in plasma than in ACE-DD subjects (Costerousse *et al.*, 1993; Soubrier *et al.*, 1993a,b; Cambien and Soubrier, 1995).

Changes in the level of ACE have been studied in a variety of conditions. Kinetics of BK hydrolysis show that it has a much lower K_m and a higher specificity constant than angiotensin I (Jaspard *et al.*, 1993; Skidgel and Erdös, 1993), and it follows that changes in plasma ACE levels are more likely to affect kinin inactivation than angiotensin I conversion. Under normal conditions, plasma ACE is very likely to originate from vascular endothelial cells (Skidgel and Erdös, 1993). In sarcoidosis, the lymph nodes contain a high concentration of ACE, which, when released, raises the enzyme level in the circulation (Lieberman, 1974, 1985; Bunting *et al.*, 1987; Silverstein *et al.*, 1976, 1979; Grönhagen-Riska, 1979). Other granulomatous diseases such as Gaucher's disease or leprosy can also elevate circulating ACE (Silverstein *et al.*, 1978; Dhople *et al.*, 1985; Lieberman, 1985). ACE is very low in monocytes and macrophages, but it can be induced by glucocorticosteroids (Friedland *et al.*, 1978), although its level decreases in plasma of sarcoidosis patients treated with the hormone. Glucocorticosteroids also elevate ACE concentrations in endothelial cells (Mendelsohn *et al.*, 1982). Thyroid hormones markedly affect plasma ACE levels (Reiners *et al.*, 1988), which are increased by hyperthyroidism and reduced by hypothyroidism (Grönhagen-Riska *et al.*, 1985; Brent *et al.*, 1984). In Addison's disease, and in silicosis, asbestosis and berylosis, ACE levels are elevated (Falezza *et al.*, 1985; Bunting *et al.*, 1987). Paradoxically, chronic administration of an ACE inhibitor leads to a higher ACE level in plasma (Fyhrquist *et al.*, 1983). In the brain, where it is localized on cell membranes, ACE concentration is lower in Huntington's chorea (Butterworth 1986; Arregui *et al.*, 1978).

2.6.1 Acute Lung Injury

In the lungs, because most circulating ACE is presumably released from vascular endothelial cells, injury to the lungs affects ACE levels in plasma. In acute lung injury there is an early increase in blood ACE (Heck and Niederle, 1983), and the enzyme also appears in pleural effusions and lavage fluid in perfused lungs (Igic *et al.*, 1972, 1973; Dragovic *et al.*, 1993). In malignant lung tumors and in leukemia, ACE activity in plasma is lower than normal (Heck and Niederle, 1983; Schweisfurth *et al.*, 1985a,b). Blood levels of ACE also tend to be lower in Hodgkin's disease and multiple myeloma (Romer and Emmertsen, 1980). In acute respiratory distress syndrome (ARDS), ACE activity decreases in serum (Bedrossian *et al.*, 1978; Johnson *et al.*, 1985b). Studies with monoclonal antibodies raised against somatic ACE suggested that the N-domain of human ACE is immunodominant (Danilov *et al.*, 1994). Monoclonal antibodies were also raised which inhibited only the N-domain of ACE, also using BK as substrate (Danilov *et al.*, 1994). These antibodies were used in a hypoxic rat lung model of pulmonary hypertension to localize ACE, which was reduced in capillary endothelium of hypoxic animals but increased in the small muscularized pulmonary arteries (Morrell *et al.*, 1995).

2.7 HYDROLYSIS OF KININS BY ACE

2.7.1 Bradykinin

The degree of participation of the two active sites of ACE in BK hydrolysis was established using mutant and wild-

type recombinant forms of the enzyme. In the mutants, one active site was eliminated by deletion or by mutation of the two zinc-binding histidine residues (Jaspard *et al.*, 1993). In agreement with previous findings, BK was cleaved by the sequential removal of the C-terminal dipeptides to yield BK_{1-7} (Fig. 7.1) and BK_{1-5}. BK is a preferred substrate of ACE over angiotensin I, since its specificity constant (k_{cat}/K_m) is about 20 times higher (Table 7.1). The N-domain alone contributes 26% of the BK degrading activity, while the C-domain is responsible for 76% of the activity in producing BK_{1-7} (Jaspard *et al.*, 1993). The C-domain active site is more dependent for its activation on Cl^-. Hydrolysis of angiotensin I by the Cl^-sensitive C-domain was activated at optimal Cl^- concentration about 100-fold while, under similar conditions, that of BK was activated only five-fold. The k_{cat}/K_m for BK is 3.6 times higher with the C-domain than with the N-domain (Jaspard *et al.*, 1993; Table 7.1). Inactivation of BK was investigated further using a naturally occurring shorter version of ACE containing only the N-domain. BK was hydrolyzed about twice as fast by the C-domain (germinal ACE) than by the N-domain (Deddish *et al.*, in preparation).

2.7.2 DesArg^9-Bradykinin

Although ACE is frequently called a peptidyl dipeptidase, it can release a C-terminal tripeptide from desArg^9-BK (Fig. 7.2), the latter being a product of carboxypeptidase N or M (Inokuchi and Nagamatsu, 1981). The k_{cat} of the tripeptide release is higher than the cleavage of the C-terminal dipeptide from BK, but the K_m of desArg^9-BK is much higher than that of BK, resulting in a much lower k_{cat}/K_m for desArg^9-BK (Inokuchi and Nagamatsu, 1981; Oshima *et al.*, 1985). ACE also liberates protected C-terminal di- and tripeptides (e.g., Gly-Leu-Met-NH_2) of substance P and even the protected N-terminal tripeptide (<Glu-His-Trp) of luteinizing hormone releasing-hormone (LHRH; Skidgel and Erdös, 1993). This N-terminal tripeptide is cleaved mainly by the N-domain (Deddish *et al.*, 1994).

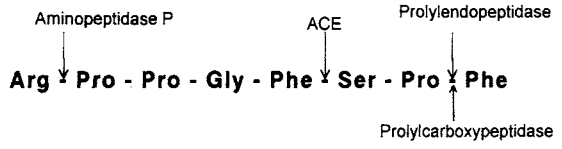

Figure 7.2 Peptide bonds in desArg^9-bradykinin cleaved by various peptidases. Arrows show the primary site of cleavage. Although it has not been determined, it is likely that neutral endopeptidase 24.11 cleaves the Gly^4-Phe^5 bond in desArg^9-bradykinin, as this bond is hydrolyzed in bradykinin under prolonged incubation conditions (Gafford *et al.*, 1983). Possibly, meprin, endopeptidase 24.15 and endopeptidase 24.16 also cleave desArg^9-bradykinin at the same sites as in bradykinin (see Fig. 7.1), but this has not been reported.

2.8 INHIBITION OF ACE AND POTENTIATION OF THE EFFECTS OF ENDOGENOUS BRADYKININ

2.8.1 Effects Attributed to Inhibition of Bradykinin Degradation

The potentiation of BK-induced effects *in vitro* and *in vivo* by inhibitors of kininases appeared initially to be due solely to prolongation of the half-life of this peptide, which is so readily degraded (Erdös *et al.*, 1963; Frey *et al.*, 1968; Erdös, 1966). However, the widespread clinical use of ACE inhibitors (Gavras *et al.*, 1978; Gavras and Gavras, 1987, 1993), along with both the development of angiotensin II receptor antagonists (Timmermans and Smith, 1994), and B_1 and B_2 kinin receptor antagonists (Regoli and Barabé, 1980; Kyle and Burch, 1993; Bhoola *et al.*, 1992; Linz *et al.*, 1995), led to renewed interest in the role of kinins as mediators of ACE inhibition. (**Editor's note:** The role of endogenous kinins in the therapeutic effects of ACE inhibitors is reviewed in Chapter 19 of this volume.)

It is unlikely that ACE inhibitors exert their major effects by raising the level of circulating kinins (Bönner, 1995). Rather these drugs likely inhibit breakdown of locally formed kinins (Carretero and Scicli, 1989, 1995). ACE is inhibited by a variety of compounds *in vitro*. Reagents which react with the Zn cofactor of the enzyme, (e.g., EDTA, *o*-phenanthroline, SH compounds), snake venom peptides, and the active form of clinically used inhibitors (e.g., enalaprilat) belong here (Erdös and Yang, 1970; Erdös, 1979). Circulating blood plasma can also inhibit ACE; the inhibition appears to be abolished by dilution of plasma (Erdös, 1979). ACE substrates can also be competitive inhibitors, and even the N-terminal tripeptide (Arg-Pro-Pro) of BK inhibits ACE *in vitro* (Oshima and Erdös, 1974). From studies of the sequence of snake venom inhibitors, synthetic ACE inhibitors were designed to retain the C-terminal proline (Kato and Suzuki, 1970; Erdös, 1979; Cushman and Ondetti, 1980; Patchett and Cordes, 1985; Wyvratt and Patchett, 1985).

Because of their two actions in blocking both angiotensin II release and preventing the inactivation of BK, the effects of ACE inhibitors may be interpreted in different ways. This issue is especially relevant when considering the experimental and therapeutic effects of ACE inhibitors in cardiovascular diseases (CONSENSUS, 1987; Gavras and Gavras, 1987, 1993; Pfeffer; 1993, 1995; Parratt, 1994; Ambrosioni *et al.*, 1995). In the coronary arteries, angiotensin II is a vasoconstrictor and, because of its mitogenic actions, causes cell proliferation in subendothelial tissues. In contrast, BK is a vasodilator and antiproliferative, at least in the rat (Swartz *et al.*, 1980; Farhy *et al.*, 1992; Sunman and Sever, 1993; Scicli, 1994; Hartman, 1995; Margolius, 1995; Auch-Schwelk *et al.*, 1993; De Meyer *et al.*, 1995). Enalapril

lowers the blood pressure of hypertensive rats; this effect is partially blocked by the administration of a BK receptor antagonist (Carbonell *et al.*, 1988). Captopril enhances skin microvascular blood flow; this was attributed to NO and prostaglandins released by BK (Warren and Loi, 1995). Thus, ACE inhibitors may improve coronary circulation by two mechanisms. BK receptors are present in heart muscles; B_2-type high affinity BK receptors were discovered in rat cardiomyocytes which are coupled to IP_3 production by a G-protein (Minshall *et al.*, 1995a). In hypertensive rats, enalapril lowers the blood pressure, an effect that is blocked partially by a BK receptor antagonist (Carbonell *et al.*, 1988).

In spontaneously hypertensive rats, high doses of ACE inhibitors prevent the development of hypertension and left ventricular hypertrophy, effects which were abolished by a B_2 receptor antagonist, Hoe 140 (Gohlke *et al.*, 1994a,b). ACE inhibitors diminished neointima formation induced by balloon injury to the rat carotid artery and endogenous kinins contribute to this effect (A.G. Scicli, personal communication). The antihypertrophic effects of ACE inhibitors may be independent of the decrease in blood pressure, and kinin receptor antagonists do not reverse this effect of ACE inhibitors (Scicli, 1994). ACE inhibitors, indeed, may prevent wall thickening in rat heart after restenosis but not the neointimal formation in other species (Shaw *et al.*, 1995).

ACE inhibitors reduced myocardial infarct size after myocardial ischemia and reperfusion injury (Liu *et al.*, 1996), but angiotensin II receptor antagonists are not effective, suggesting that kinins were responsible. ACE inhibitors also ameliorated cardiac arrhythmias induced by digoxin or reperfusion, in part via endogenous BK (Linz *et al.*, 1995). On the other hand, prevention of cardiac myocyte necrosis and coronary vascular damage induced by angiotensin II in the rat, was blocked by lisinopril as well as by an angiotensin II receptor antagonist (Kabour *et al.*, 1995). In one study, administration of an NO synthase inhibitor resulted in the development of hypertension and cardiac and renal insufficiency, which was prevented by ramiprilat. The beneficial effect was attributed to increased BK and prostaglandin production (Hropot *et al.*, 1994).

ACE inhibitors, by enhancing the effects of BK on energy metabolism, may improve cardiac metabolism. Dietze (1982) showed that BK potentiates glucose uptake, in part via the release of prostaglandins. ACE inhibitors reduce lactate release from the heart (Linz *et al.*, 1995) by enhancing the effects of BK. The metabolic functions of the guinea-pig heart were improved by an ACE inhibitor, which decreased lactic acid release and increased intracellular glutathione, and these effects were attributed to endogenous BK (Massoudy *et al.*, 1994). Captopril increased glucose utilization in patients and ACE inhibitors can cause hypoglycemia (Torlone and Bolli, 1991). The beneficial effects of ACE inhibition in reducing hyperfiltration in experimental diabetes of rats was attributed to kinins as mediators (Komers and Cooper, 1995).

The roles of BK potentiation in the clinical benefits, or side effects of acute or chronic administration of ACE inhibitors are still being explored. ACE inhibitors given soon after myocardial infarction reduced mortality and the development of severe heart failure (Gavras and Gavras, 1993; Hall *et al.*, 1994; Pfeffer, 1995). Whether or not coughing, a prominent side effect of ACE inhibitors (Israili and Hall, 1992; Semple, 1995) is caused by prolongation of the half-life of BK or by potentiation of BK's effects is unclear. BK may also be involved in a rare but serious side effect of ACE inhibitors, angioneurotic edema (Slater *et al.*, 1988).

2.8.2 Effects Not Attributed to Inhibition of Bradykinin Degradation

The potentiation of the effects of BK by ACE inhibitors go beyond protecting BK from degradation. Decades of research have yielded several compounds that amplify effects of BK, at least in guinea-pig isolated ileum (Vogel *et al.*, 1970). These agents are unrelated in structure, ranging from snake venom peptides and sulfhydryl compounds, known inhibitors of ACE, to proteases and fibrinopeptides, which do not inhibit ACE (Gladner *et al.*, 1963; Edery, 1964, 1965). This issue recently gained particular importance when ACE inhibitors were demonstrated to improve functions in the failing heart via a mechanism that was attributed to the potentiation of the *action* of BK and *not* inhibition of its metabolism (Scicli, 1994; Linz *et al.*, 1995). A detailed discussion is beyond the scope of this review but a brief summary of the findings follows.

The evidence supporting the hypothesis that potentiation of the actions of BK is not necessarily due solely to inhibition of its enzymatic breakdown has been the subject of several publications (e.g., Paegelow *et al.*, 1976). In guinea-pig ileum or heart, the peptide is inactivated slowly, while the potentiation by ACE inhibitors can be almost instantaneous and is unlikely, therefore, to be due to prolonging the half-life of BK (Ufkes *et al.*, 1977). In isolated guinea-pig atrium, ACE inhibitors potentiated effects of BK even when an ACE-resistant BK analog was used (Auch-Schwelk *et al.*, 1993; Minshall *et al.*, 1995b). ACE inhibitors also potentiated the inotropic effects of BK even when the ACE was inhibited completely by sequestration of its metal cofactor (Minshall *et al.*, 1995b).

An ACE inhibitor also enhanced the release of endothelium-derived hyperpolarizing factor by either BK or a metabolically stable BK analog (Ibrahim *et al.*, 1995; Mombouli and Vanhoutte, 1995). This action was attributed to a possible interaction of ACE inhibitors with BK receptors. ACE inhibitors enhance the relaxation of isolated coronary artery in response to BK and this was endothelium-dependent (Auch-Schwelk *et al.*, 1993). Because responses to a degradation-resistant

analog of BK were also potentiated by an ACE inhibitor and no hydrolysis of BK was observed *in vitro*, the phenomenon was attributed to a "potentiation" of the effect of BK rather than inhibition of its degradation. It remains to be established, however, whether ACE inhibitors affect BK receptors directly or via interaction with ACE, which may be in close physical proximity, and thereby enhance the action of BK independent of prolongation of its half-life.

3. *Neutral Endopeptidase 24.11*

Neutral endopeptidase 24.11 (neprilysin, NEP) is a zinc-metallopeptidase with a single active site containing the canonical HEXXH sequence (Erdös and Skidgel, 1989; Skidgel, 1993; Howell *et al.*, 1994). The primary sequence is well conserved among species (Devault *et al.*, 1987), there being only six nonconserved differences between the human and rat NEP (Malfroy *et al.*, 1987, 1988). The enzyme is a transmembrane, single-chain protein of 742 amino acids but, in contrast to ACE, is bound via an uncleaved N-terminal signal peptide (Roy *et al.*, 1993).

3.1 LOCALIZATION

NEP is distributed widely but, in contrast to ACE, its expression in vascular endothelial cells is low (Johnson *et al.*, 1985a; Llorens-Cortes *et al.*, 1992; Howell *et al.*, 1994), and varies within vascular beds (Graf *et al.*, 1995). Epithelial cells, especially in microvillar structures, are rich in NEP (Johnson *et al.*, 1985a; Turner, 1987; Ronco *et al.*, 1988). As with ACE, these include the proximal tubules (Kerr and Kenny, 1974), placenta, (Johnson *et al.*, 1984), intestine, or the choroid plexus (Turner, 1987; Ronco *et al.*, 1988). Its distribution in the brain has been investigated in detail, possibly because neuropeptides, such as enkephalins and substance P, are among its substrates (Turner, 1987). Owing to its wide distribution, actions which are relevant *in vivo* can be different in each organ with the different substrates. As with ACE, the relevance of the high concentration of NEP in the male genital tract, especially in prostate glands (Erdös *et al.*, 1985) is not known. NEP, under the name, "common acute lymphoblastic leukemia antigen" (CALLA or CD10) (Letarte *et al.*, 1988; LeBien and McCormack, 1989) is present in lymphoblasts but is absent from mature lymphocytes. In contrast, it is found in neutrophils but absent from progenitor cells (Connelly *et al.*, 1985; Painter *et al.*, 1988).

3.2 ENZYMATIC PROPERTIES

NEP cleaves peptides at the N-termini of hydrophobic amino acids, although the molecular mass of substrates does not usually exceed 3 kDa. NEP is a second kininase II that releases the C-terminal Phe^8-Arg^9 of BK (Gafford *et al.*, 1983). Although it was discovered as an endopeptidase that cleaves the B-chain of insulin (Kerr and Kenny, 1974), many active peptide substrates for NEP have been described subsequently, and include enkephalins (Schwartz *et al.*, 1985), endothelin (Vijayaraghavan *et al.*, 1990), atrial natriuretic peptide, substance P and a chemotactic peptide (Connelly *et al.*, 1985; Erdös and Skidgel, 1989).

As noted above, NEP cleaves BK at the Pro^7-Phe^8 bond (Fig. 7.1) (Almenoff *et al.*, 1981), which was first shown qualitatively by high pressure liquid chromatography, although prolonged incubation also results in the hydrolysis of the Gly^4-Phe^5 bond (Gafford *et al.*, 1983). By the use of purified human renal NEP, the kinetics of hydrolysis were established (Gafford *et al.*, 1983; Table 7.1). The k_{cat} for BK is higher with NEP than with ACE but, because of the higher K_m (120 μM vs 0.18 μM), the specificity constant, k_{cat}/K_m, of NEP is lower (40 vs 3,667 $\mu M^{-1}\ min^{-1}$).

Sites where NEP may be an important kininase include the epithelial cells of the respiratory tract (Johnson *et al.*, 1985a; Dusser *et al.*, 1988), skeletal muscles (Dragovic *et al.*, 1996b), neutrophils (Connelly *et al.*, 1985; Painter *et al.*, 1988; Skidgel *et al.*, 1991b), renal proximal tubules (Skidgel *et al.*, 1987b; Ura *et al.*, 1987) and possibly human coronary vessels (Graf *et al.*, 1995). Inhibitors of NEP (e.g., thiorphan, phosphoramidon and candoxatril) are thus useful tools in exploring the functions *in vivo* of this peptidase (Schwartz *et al.*, 1985; Bralet *et al.*, 1991; Elsner *et al.*, 1992; Nadel, 1992, 1994; Bertrand *et al.*, 1993; Schilero *et al.*, 1994).

Because of the antiproliferative effects of BK (Farhy *et al.*, 1992), the high concentration of NEP in solid malignant tumors may indicate that BK is a substrate in those cells. Transplanted malignant tumors of rat liver, SK HEP1 malignant human liver cells (Dragovic *et al.*, 1994), and primary liver tumors (Dragovic *et al.*, 1996a) contain as much as 1,000 times more NEP than noncancerous tissues (Dragovic *et al.*, 1994).

3.2.1 Soluble NEP

NEP levels in circulating blood plasma are normally very low, but increase 60–80-fold in ARDS with septic pneumonia (Johnson *et al.*, 1985b). Chronic cholestasis is another condition that enhances plasma NEP activity (Swan *et al.*, 1993). In contrast to plasma, the level of NEP in amniotic fluid is high (Spillantini *et al.*, 1990). Ura and colleagues (1987) found that most kininase activity in rat urine is attributable to high NEP activity, and injection of an NEP inhibitor enhanced the diuretic and natriuretic effects of kinins. These effects could be mediated in part by an interaction of atrial natriuretic peptide and kinins in the rat kidney (Smits *et al.*, 1990; Bralet *et al.*, 1991). NEP was present in high concen-

tration in the developing edema of the perfused rat lung, while its level in the perfusate was low (Dragovic *et al.*, 1993). NEP activity increased in the urine after proximal tubular injury and in serum in end-stage renal disease (Deschodt-Lanckman *et al.*, 1989; Nortier *et al.*, 1993).

3.3 INHIBITION OF NEP AND ITS ROLE AS A KININASE

Inhibition of NEP in rat bronchial epithelium markedly enhances bronchoconstriction induced by substance P and BK (Bertrand *et al.*, 1993). The BK-induced bronchoconstriction (Barnes, 1987) in asthmatic patients is enhanced by NEP inhibitors (Crimi *et al.*, 1995). Indirect actions of BK are also influenced by NEP inhibition. For example, BK releases substance P from nerve endings, which is then cleaved by NEP (Bhoola *et al.*, 1992; Barnes, 1994; Nadel, 1994; Skidgel, 1994). BK stimulates airway ciliary activity via prostaglandin E_2 release in rabbit and this effect is also regulated by NEP (Tamaoki *et al.*, 1989).

Microvascular leakage in guinea-pig airways was potentiated by both ACE and NEP inhibition (Lötvall, 1990; Lötvall *et al.*, 1990). In the rat lung, combined inhibition of NEP and ACE enhanced the effect of a subthreshold dose of BK in the development of edema (Dragovic *et al.*, 1993). Injecting BK into the left ventricle of rats increased the blood flow in the microcirculation of airways (Yamawaki *et al.*, 1994) and this, too, was potentiated by phosphoramidon, an NEP inhibitor; thus, besides ACE, NEP participates in the inactivation of BK there. In the rat heart, sensory nerve stimulation enhanced myocardial blood flow that was abolished by a BK receptor blocker but potentiated by phosphoramidon (Piedimonte *et al.*, 1994).

Because of the increased reactivity to the combined administration of inhibitors of two peptidases, several compounds that inhibit both NEP and ACE, or ACE and aminopeptidase are now available (Gros *et al.*, 1991; Bralet *et al.*, 1994; French *et al.*, 1994; Flynn *et al.*, 1995; Gonzalez-Vera *et al.*, 1995). For example, a dual inhibitor of ACE and NEP, administered intravenously (i.v.) to rats, potentiates the hypotensive effect of BK more than the action of an inhibitor specific to either enzyme alone (French *et al.*, 1995). Canine renal responses to BK are enhanced by combined administration of ACE and NEP inhibitors (Seymour *et al.*, 1993, 1994). This combination lowered arterial blood pressure and vascular resistance in nonanesthetized dogs (Seymour *et al.*, 1994). Co-administration of inhibitors of ACE and aminopeptidase P is more effective in reducing the blood pressure in hypertensive rats than inhibition of either enzyme alone (A.G. Scicli *et al.*, personal communication).

4. *Hydrolysis of Bradykinin by Carboxypeptidases*

Carboxypeptidases catalyze the hydrolysis of the C-terminal peptide bond in peptides and proteins. Their specificity is largely determined by the C-terminal and/or penultimate residue of the substrate (Skidgel, 1996). Carboxypeptidases are subdivided into two general groups; the serine carboxypeptidases and the metallocarboxypeptidases. Any carboxypeptidase that removes the C-terminal Arg of BK can be termed "kininase I" (Fig. 7.1). For example, the active subunit of carboxypeptidase N has 41% sequence identity with carboxypeptidase M and both enzymes cleave BK. Although deamidase/cathepsin A cleaves the C-terminal Arg of BK (Fig. 7.1) and, therefore, may be classified as a kininase I, it is a serine carboxypeptidase with no homology to carboxypeptidase M or N (Jackman *et al.*, 1990; Skidgel, 1996).

4.1 METALLOCARBOXYPEPTIDASES

Metallocarboxypeptidases catalyze peptide hydrolysis through a mechanism that requires the participation of a tightly bound zinc atom as an essential cofactor (Skidgel, 1996). The metallocarboxypeptidases can be subdivided further into two categories based on their substrate specificities: carboxypeptidase A-type enzymes prefer hydrophobic C-terminal amino acids, whereas the carboxypeptidase B-type enzymes cleave only C-terminal Arg or Lys (Skidgel, 1988). Since the kinins contain a C-terminal Arg, however, only carboxypeptidase B-type enzymes cleave them.

4.1.1 General Characteristics

Because of the essential function of zinc in catalysis, these carboxypeptidases are all inhibited by metal chelating agents such as 1,10-phenanthroline (Skidgel, 1996). All of the B-type carboxypeptidases can be inhibited relatively specifically by small synthetic arginine analogs such as DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) and guanidinoethylmercaptosuccinic acid (GEMSA) (Plummer and Ryan, 1981; Skidgel, 1991, 1996).

Depending upon the enzyme employed, they inhibit in the low micromolar to nanomolar range. The pH of the incubation and the form of the enzyme can also affect the affinity for these inhibitors. For example, the IC_{50} of GEMSA for membrane bound carboxypeptidase M at pH 5.5 is 100-fold lower than for the solubilized, purified enzyme at pH 7.5 (Deddish *et al.*, 1989; Skidgel, 1991).

Replacement of zinc in the active center with other divalent cations, most notably by cobalt or cadmium, alters the activity of these enzymes. For example, Co^{2+} increases carboxypeptidase activity by up to ten-fold, depending on the source of enzyme or the substrate

(Folk and Gladner, 1960, 1961; Marinkovic *et al.*, 1977; Skidgel, 1988; Deddish *et al.*, 1989). The pH is an additional factor that affects activation by Co^{2+} (Deddish *et al.*, 1989; Skidgel, 1991); it activates the enzyme more at a low pH. As with carboxypeptidase N (Skidgel *et al.*, 1984b), stimulation of peptidase activity by Co^{2+} is due to an increase in the V_{max}, as the K_m increases in the presence of this metal ion. In contrast, Cd^{2+} inhibits the peptidase activity of the metallocarboxypeptidases with the exception of carboxypeptidase U (plasma carboxypeptidase B), which is stimulated (Skidgel, 1996).

Based on the primary sequences, mammalian metallocarboxypeptidases fall clearly into two groups; those with a high sequence identity with the pancreatic carboxypeptidases and the so-called regulatory carboxypeptidases. Pancreatic carboxypeptidases A and B, mast cell carboxypeptidase A and carboxypeptidase U (Skidgel, 1996) form the first group and carboxypeptidases N, M and E the second group. Within each group, there is significant sequence similarity (40–58%) whereas between groups, the identity is much lower (14–20%). These data, as well as conservation of active site residues, indicate that the metallocarboxypeptidases arose from the same ancestral gene which duplicated and diverged to evolve into two separate precursor genes, one of which gave rise to the pancreatic carboxypeptidase-like enzymes and the other to regulatory carboxypeptidases M, N and E (Tan *et al.*, 1989a; Avilés *et al.*, 1993).

4.1.2 Carboxypeptidase N

The discovery that a plasma carboxypeptidase inactivates BK was the first demonstration that an endogenous carboxypeptidase could regulate the activity of a peptide hormone (Erdös and Sloane, 1962). Although the enzyme has been given several names (e.g., anaphylatoxin inactivator, creatine kinase conversion factor, plasma carboxypeptidase B, arginine carboxypeptidase, lysine carboxypeptidase, protaminase), the original designation, carboxypeptidase N, is used here.

4.1.2.1 Physical and Structural Properties

Size estimates for purified native human carboxypeptidase N range from about 270 to 330 kDa, depending on the method used but 280 kDa is most frequently cited (Oshima *et al.*, 1975; Plummer and Hurwitz, 1978; Levin *et al.*, 1982). Under denaturing conditions, it dissociates into three major bands of 83 kDa, 55 kDa and 48 kDa (Plummer and Hurwitz, 1978; Levin *et al.*, 1982). The 83 kDa protein is a noncatalytic subunit, whereas the 55 kDa and 48 kDa proteins represent two forms of the same active subunit (Levin *et al.*, 1982; Skidgel, 1995). Carboxypeptidase N, therefore, is a tetrameric enzyme comprised of two heterodimers, each heterodimer containing one catalytic and one noncatalytic 83 kDa subunit. The 83 kDa subunit is heavily glycosylated, (about 28% by weight), the carbohydrate composition being typical of proteins containing Asn-linked complex carbohydrate chains (Plummer and Hurwitz, 1978; Levin *et al.*, 1982). In contrast, the active subunit lacks carbohydrate.

As revealed by molecular cloning, the 50 kDa subunit has sequence similarities to other metallocarboxypeptidases ranging from 14% to 49% (Gebhard *et al.*, 1989). Cloning and sequencing of the 83 kDa subunit, however, revealed that it encodes a 59 kDa protein with no sequence similarity to the 50 kDa active subunit or other carboxypeptidases (Tan *et al.*, 1990). Consistent with its high carbohydrate content, the sequence contains seven potential Asn-linked glycosylation sites and a serine/threonine rich region that may be a site for attachment of O-linked carbohydrate. The most exceptional feature is a domain comprising over half of the protein that contains 12 leucine-rich tandem repeats of 24 amino acids each (Tan *et al.*, 1990; Skidgel and Tan, 1992). This pattern was initially described in the sequence of the leucine-rich α_2-glycoprotein, although a variety of other mammalian proteins contain it (Tan *et al.*, 1990; Skidgel and Tan, 1992; Kobe and Deisenhofer, 1993). The leucine-rich repeat region is likely to be critical for the binding function of these proteins (Kobe and Deisenhofer, 1993). Probably the leucine-rich repeat region in the 83 kDa subunit mediates its interaction with the 50 kDa active subunit to form a heterodimer, and the N- and C-terminal domains of the 83 kDa subunit link the two heterodimers to form the tetramer (Tan *et al.*, 1990; Skidgel and Tan, 1992).

Because of its lack of carbohydrate, small size and relative instability at 37°C (Levin *et al.*, 1982; Skidgel, 1988), the active subunit of carboxypeptidase N by itself probably would not exist in the circulation for long. *In vitro*, the 83 kDa subunit stabilizes the active subunit at 37°C and at low pH (Levin *et al.*, 1982). This indicates that the 83 kDa subunit, although lacking enzymatic activity, is important in carrying and stabilizing the active subunit in the blood.

4.1.2.2 Enzymatic Properties

Carboxypeptidase N cleaves a variety of substrates containing C-terminal Arg or Lys, generally cleaving Lys faster than Arg, although the penultimate residue also plays an important role, alanine being preferred in many cases (Skidgel, 1995). The pH optimum of carboxypeptidase N is in the neutral range (7.5) and it retains minimal activity at pH 5.5 (Erdös *et al.*, 1964; Deddish *et al.*, 1989). However, Co^{2+} still activates the enzyme at pH 5.5 to a surprising 156% of the activity at pH 7.5 in the absence of Co^{2+} (Deddish *et al.*, 1989). While most of the enzymatic properties of the isolated catalytic 50 kDa subunit (Levin *et al.*, 1982) agree with those of the intact 280 kDa tetramer, the 83 kDa subunit can affect allosterically the interaction of the enzyme with some substrates including the anaphylatoxin C3a, and inhibitors such as protamine (Skidgel *et al.*, 1986; Tan *et al.*, 1989b).

4.1.2.3 *Localization*

Carboxypeptidase N is synthesized in the liver and released into the circulation where it is present at a relatively high concentration of approximately 30 μg/ml (10^{-7} M) (Erdös, 1979). Little carboxypeptidase N activity is found in liver (Oshima *et al.*, 1975), probably because the enzyme is not stored, but secreted soon after synthesis. Apparently, no other cells or organs synthesize the enzyme, as Northern blot analysis of various organs gave negative results (Tan and Skidgel, unpublished). Although it was reported that carboxypeptidase N immunoreactivity is present on the cell membrane of cultured bovine pulmonary arterial endothelial cells (Ryan and Ryan, 1983), it was not determined whether the cells synthesize the enzyme or, more likely, take it up from the serum in the medium. In contrast, cell membranes of cultured human pulmonary arterial endothelial cells have high levels of carboxypeptidase M, but no detectable carboxypeptidase N (Nagae *et al.*, 1993).

4.1.2.4 *Role of Carboxypeptidase N as a Kininase*

Carboxypeptidase N was discovered as a kininase that cleaved the C-terminal Arg^9 from BK (Fig. 7.1). Although the k_{cat} value for BK is lower than other naturally occurring substrates (Table 7.1), BK has the lowest K_m (19 μM) of all the peptide substrates tested (Skidgel, 1995). Even though the ability of carboxypeptidase N to cleave kinins *in vitro* is well documented (Erdös, 1979), its contribution to the degradation of circulating kinins *in vivo* is probably of secondary importance as BK is rapidly inactivated by ACE during a single passage through the pulmonary circulation (Vane, 1969; Erdös, 1979). Also, although the rate of degradation of circulating BK would be slower, this pathway becomes more significant in patients treated with ACE inhibitors. Levels of circulating kinins may not be relevant to their physiological actions, which are thought to be localized in tissues (Carretero and Scicli, 1989). Rather, they likely represent "spillover" of kinins generated at local sites. Nevertheless, should kinin concentrations increase significantly in the blood, they can have undesirable effects. Thus, blood-borne enzymes may be important in preventing buildup of endogenous peptides in the circulation. The presence of carboxypeptidase N in blood in a relatively high concentration provides evidence that this enzyme may fulfill this function. Further evidence is the observation that no human subjects tested have been found to lack the enzyme completely and even patients with low enzyme levels are rare (Erdös *et al.*, 1965; Mathews *et al.*, 1980). Indeed, genetically low blood levels of carboxypeptidase N (about 20% of normal), owing to decreased hepatic synthesis, were associated with repeated attacks of angioedema in one patient, possibly due to the increased half-life of kinins and/or anaphylatoxins (Mathews *et al.*, 1980, 1986). Conditions that affect hepatic plasma protein synthesis also alter plasma carboxypeptidase N levels, and include cirrhosis of the liver, which causes a decrease, or pregnancy, which causes an increase (Erdös *et al.*, 1965). In a variety of diseases (e.g., cardiovascular disease, diabetes, allergic conditions), there are no changes in carboxypeptidase N levels (Erdös *et al.*, 1965; Mathews *et al.*, 1980), although elevations in enzyme level have been noted in certain cancers, and in the blood and synovial fluid of arthritic patients (Erdös *et al.*, 1965; Mathews *et al.*, 1980; Schweisfurth *et al.*, 1985b; Chercuitte *et al.*, 1987). The relationship of carboxypeptidase N levels to these disease states, if any, is not known.

The evidence for the protective function of carboxypeptidase N in humans is, by necessity, somewhat indirect. Nevertheless, the protamine-reversal syndrome is one condition where low carboxypeptidase N activity may be involved. Protamine is given routinely to neutralize the antithrombotic effects of heparin after extracorporeal circulation. In some patients, this can trigger a catastrophic reaction consisting of pulmonary vasoconstriction, bronchoconstriction and systemic hypotension (Lowenstein *et al.*, 1983; Morel *et al.*, 1987). This reaction has been attributed to the release of thromboxane, and the generation of anaphylatoxins and kinins subsequent to the activation of the complement cascade and factor XII, which activates plasma kallikrein (Colman, 1987; Morel *et al.*, 1987). As protamine is a potent inhibitor of carboxypeptidase N (Tan *et al.*, 1989b), potentially decreased degradation of anaphylatoxins and kinins may contribute to this syndrome. In addition, the carboxypeptidase N concentration decreases to about 50% of normal after initiation of cardiopulmonary bypass, due primarily to dilution of the blood (Rabito *et al.*, 1992). However, the fact that this syndrome is relatively rare (incidence is approximately 1%) indicates that other factors are involved. Because heparin binds protamine and reverses the inhibition of carboxypeptidase N (Tan *et al.*, 1989b), only when protamine is given in excess would a problem develop. In agreement with this possibility, administration of heparin reversed protamine reactions in two patients and was hypothesized to be due to reactivation of carboxypeptidase N (Lock and Hessel, 1990). In addition, the data of Mathews *et al.* (1980) indicate that carboxypeptidase N levels of 20% of normal or greater are sufficient for a protective role.

Most of the actions of BK are mediated via binding and activation of B_2 receptors (Bhoola *et al.*, 1992). However, cleavage of BK by carboxypeptidase N yields $desArg^9$-BK, a specific agonist at B_1 receptors, which stimulates a variety of proinflammatory cellular responses (see Chapters 2, 8, 9 and 13 of this volume). The B_1 receptor system is upregulated in response to injury or inflammation, and may be part of the acute phase reaction (Bhoola *et al.*, 1992; see Chapter 8). For example, many isolated tissues respond to $desArg^9$-BK only after incubation for several hours (DeBlois *et al.*, 1991; Bhoola *et al.*, 1992). Noxious or pro-inflammatory

stimuli, such as Triton X-100, endotoxin, or interleukins-1 and -2, induce the expression of B_1 receptors (Bhoola *et al.*, 1992; Crecelius *et al.*, 1986; DeBlois *et al.*, 1991), and this is inhibited by glucocorticosteroids (DeBlois *et al.*, 1988). Thus, conversion of BK to desArg^9-BK by carboxypeptidase N produces an agonist for B_1 receptors and may play an important role in inflammatory or pathological responses. That this occurs *in vivo* is supported by the finding that blood levels of desArg^9-BK are over 3-fold higher than native BK in normotensive individuals and in patients with low-renin essential hypertension (Odya, *et al.*, 1983).

4.1.3 Carboxypeptidase M

We discovered significant carboxypeptidase B-like activity in membrane fractions obtained from human and animal tissues and cells (Johnson *et al.*, 1984; Skidgel *et al.*, 1984b). After purification and characterization of this unique enzyme, we named it carboxypeptidase "M" to denote the fact that it is membrane-bound (Skidgel *et al.*, 1989).

4.1.3.1 Physical and Structural Properties

Human carboxypeptidase M yields only a single band of 62 kDa, in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) with or without reduction, showing that it is a single-chain protein (Skidgel *et al.*, 1989). A slightly higher value (73 kDa), obtained in gel filtration in the presence of CHAPS, is likely to be caused by the binding of the enzyme to detergent micelles and also to its glycoprotein nature (Skidgel *et al.*, 1989). Because carboxypeptidase M is a glycoprotein, it binds tightly to concanavalin A–Sepharose and its mass is reduced to 47.6 kDa by chemical deglycosylation (Skidgel *et al.*, 1989). These data indicate a 23% carbohydrate content by weight, in agreement with the presence of six potential Asn-linked glycosylation sites in the deduced protein sequence (Tan *et al.*, 1989a).

In subcellular fractions of cells or tissues, most carboxypeptidase M is firmly membrane bound (Skidgel *et al.*, 1984b, 1989; Skidgel, 1988). However, the primary sequence does not contain a true hydrophobic transmembrane spanning region (Tan *et al.*, 1989a). The extreme C-terminus has a weakly hydrophobic region of 15 amino acids, similar to other proteins that are membrane bound via a glycosylphosphatidylinositol (GPI) anchor (Low, 1987). Indeed, carboxypeptidase M can be released from membrane preparations by bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), a characteristic feature of GPI-anchored proteins (Deddish *et al.*, 1990; Tan *et al.*, 1995). Direct evidence for the presence of a GPI anchor on carboxypeptidase M was obtained by labeling cultured Madin Darby canine kidney (MDCK) cells (which have high carboxypeptidase M activity) with [^{3}H]-ethanolamine. Antiserum specific for carboxypeptidase M immunoprecipitated a single radiolabeled band from the solubilized membrane fraction, corresponding in size to that of carboxypeptidase M (Deddish *et al.*, 1990). Carboxypeptidase M is also found in soluble form in various body fluids such as urine, seminal plasma, amniotic fluid and bronchoalveolar lavage fluid (Skidgel *et al.*, 1984a,b, 1988; Dragovic *et al.*, 1995; McGwire and Skidgel, 1995). The mechanism of release of the enzyme has not been determined, but the fact that the hydrophobic portion of the anchor was removed indicates that either a protease or phospholipase is involved (Deddish *et al.*, 1990).

4.1.3.2 Enzymatic Properties

Carboxypeptidase M is activated by cobalt chloride and inhibited by *o*-phenanthroline, MGTA, GEMSA and cadmium acetate, as are most B-type carboxypeptidases. It has a neutral pH optimum and cleaves only C-terminal Arg or Lys from a variety of substrates including BK and arginine or lysine-extended opioid peptides (Fig. 7.1). Carboxypeptidase M cleaves C-terminal Arg preferentially over Lys, as demonstrated by faster cleavage of Bz-Gly-Arg than Bz-Gly-Lys, although the penultimate residue prominently affects the rate of hydrolysis (Skidgel *et al.*, 1989). Carboxypeptidase M also hydrolyzed naturally occurring peptide substrates and the kinetic constants were determined (Skidgel *et al.*, 1989). Of the substrates tested, BK (with C-terminal Phe^8-Arg^9) has the lowest K_m (16 μM) and the k_{cat}/K_m is 9.2 $\mu M^{-1}min^{-1}$ (Table 7.1). Of the synthetic substrates tested, the ester substrate (1 mM Bz-Gly-argininic acid) was cleaved fastest (102 micromoles/min/mg).

4.1.3.3 Localization

Carboxypeptidase M is found in a wide variety of tissues and cells. Northern blot analysis showed high levels of carboxypeptidase M mRNA in human placenta, lung and kidney (Nagae *et al.*, 1992). Significant amounts are present in blood vessels, intestine, brain and in peripheral nerves (Skidgel, *et al.*, 1984b, 1991a; Skidgel, 1988; Nagae *et al.*, 1992, 1993). In the lungs, immunohistochemical studies located carboxypeptidase M on the surface of type I pneumocytes and in pulmonary macrophages (Nagae *et al.*, 1993). In the brain, oligodendrocytes or astrocytes stain positively and, in peripheral nerves, the enzyme is concentrated on the outer aspects of myelin sheaths and Schwann cell membranes (Nagae *et al.*, 1992). Carboxypeptidase M is also present in soluble form in various body fluids, as noted earlier (Skidgel *et al.*, 1984a,b, 1988; McGwire and Skidgel, 1995). Recently it was discovered that monoclonal antibodies, raised against a differentiation-dependent cell surface antigen on white blood cells, are specific for carboxypeptidase M (de Saint-Vis *et al.*, 1995; Rehli *et al.*, 1995). In one study, carboxypeptidase M was almost undetectable on peripheral blood monocytes, but highly expressed after differentiation into macrophages (Rehli *et al.*, 1995). Similarly, carboxypeptidase M was present on pre-B lymphocytes, downregulated on circulating

B lymphocytes, but re-expressed on activated germinal center B cells (de Saint-Vis *et al.*, 1995).

4.1.3.4 *Role of Carboxypeptidase M as a Kininase*

Owing to its location on the plasma membranes of a wide variety of cells and tissues, where the BK receptor is also located, carboxypeptidase M is likely to regulate the local actions of BK (Skidgel, 1988, 1992). As with carboxypeptidase N, desArg^9-BK, an agonist for B_1 receptors, is produced by carboxypeptidase M (Fig. 7.1). However, because of its location on plasma membranes, carboxypeptidase M may perform this role in a local environment outside the circulation, for example, at sites of inflammation where B_1 receptors may be upregulated (Skidgel, 1992, 1996; Bhoola *et al.*, 1992).

Epidermal growth factor (EGF; urogastrone) is another substrate of carboxypeptidase M with relevance to the kinin system. EGF is a 53 amino-acid (6 kDa) peptide first isolated from mouse submaxillary gland (Carpenter and Wahl, 1990). Studies in liver and fibroblasts indicated that EGF is first cleaved by a carboxypeptidase to remove the C-terminal Arg, producing EGF_{1-52} at the cell surface or in early endosomes (Schaudies and Savage, 1986; Planck *et al.*, 1984; Renfrew and Hubbard, 1991). Recent studies showed that purified carboxypeptidase M readily converts EGF to desArg^{53}-EGF (McGwire and Skidgel, 1995). Incubation of EGF with MDCK cells, which have high carboxypeptidase M activity, resulted in rapid conversion (61% in 2 h) of EGF to desArg^{53}-EGF as the only metabolite. The hydrolysis was blocked completely by a carboxypeptidase inhibitor, MGTA. Similar results were obtained with urine or amniotic fluid where MGTA or immunoprecipitation with specific antiserum to carboxypeptidase M abolished essentially all EGF hydrolysis (McGwire and Skidgel, 1995). However, there was no difference in the mitogenic potency of EGF and desArg^{53}-EGF on MDCK cells and conversion of EGF to desArg^{53}-EGF was not required for the mitogenic effect (McGwire and Skidgel, 1995). Because carboxypeptidase M cleaves both EGF and BK, it is of interest that EGF stimulates the growth of breast stromal cells, whereas BK decreases growth and causes a concentration-dependent inhibition of EGF-stimulated DNA synthesis (Patel and Schrey, 1992). This effect of BK is mediated through B_1 receptors, implying it is first converted to desArg^9-BK in this system. EGF also potentiates the contractile response to desArg^9-BK in rabbit aortic rings (DeBlois *et al.*, 1992).

Carboxypeptidase M may be involved in inflammatory and pathological processes by virtue of regulating the activity of kinins and anaphylatoxins that mediate many inflammatory effects. The importance of the up- or downregulation of carboxypeptidase M during differentiation of monocytes to macrophages and in B lymphocytes (Rehli *et al.*, 1995; de Saint-Vis *et al.*, 1995) is not known. In the kidney, carboxypeptidase M may control the activity of kinins which are released by kallikrein liberated from the distal tubules (Scicli *et al.*, 1978). Kinins stimulate prostaglandin production, affect sodium and water excretion (Carretero and Scicli, 1989) and mediate amino-acid-induced hyperperfusion and hyperfiltration (Jaffa *et al.*, 1992). In one study, hypertensive human patients excreted significantly more kininases than normal individuals and the major kininase was a kininase I (Iimura, 1987), probably carboxypeptidase M. Thus, increased release of carboxypeptidase M into the urine could be an early sign of renal damage owing to hypertension or other diseases.

Carboxypeptidase M may also have important functions in the lung as indicated by the high level of activity present in membrane fractions from the lungs of bovines, guinea pigs, baboons, dogs, rats and humans (Chodimella *et al.*, 1991; Nagae *et al.*, 1993). Type I cells, which in immunohistochemistry stain strongly for carboxypeptidase M (Nagae *et al.*, 1993), comprise only 8% of the total cells in the lung, yet they account for 93% of the total surface area. The cells are covered with a layer of surfactant and function primarily as a thin gas-permeable membrane between the air space and capillary. The presence of carboxypeptidase M on this surface indicates a protective role. The enzyme may also be readily mobilized from the cell surface or be soluble in the surfactant layer as bronchoalveolar lavage fluid contains high levels of carboxypeptidase M (Dragovic *et al.*, 1993, 1995). The pulmonary synthesis of carboxypeptidase M or its release from the membrane may be upregulated in disease states as enzyme levels in bronchoalveolar lavage fluid were elevated almost five-fold in patients with pneumocystic or bacterial pneumonia or lung cancer (Dragovic *et al.*, 1995). Carboxypeptidase M was also released into the edema fluid of rat lung in an experimental model of lung injury (Dragovic *et al.*, 1993).

BK can cause pulmonary edema and bronchoconstriction when administered to animals. Although the carboxypeptidase inhibitor MGTA does not enhance either of these responses by itself (Ichinose and Barnes, 1990; Chodimella *et al.*, 1991; Dragovic *et al.*, 1993), it causes further potentiation of these pulmonary responses after inhibition of NEP and ACE (Chodimella *et al.*, 1991; Dragovic *et al.*, 1993), suggesting the involvement of all three enzymes in the metabolism of BK. This might be relevant to the persistent dry cough that is one of the major side effects encountered after the administration of ACE (kininase II) inhibitors to hypertensive patients (Israili and Hall, 1992; Semple, 1995). Because this can result from increased concentration of peptides such as BK in the respiratory tract (Morice *et al.*, 1987) it adds to the potential importance of carboxypeptidase M in the lungs of these patients.

Studies in guinea pigs revealed that a carboxypeptidase M-type enzyme exists in the airways, and that

MGTA enhances the noncholinergic bronchoconstrictor response to capsaicin and vagus nerve stimulation (Desmazes *et al.*, 1992). This probably involves the release of peptides from nerve endings, although BK, presumably, is not involved (Desmazes *et al.*, 1992). While the response to MGTA was attributed to inhibition of the activity in the airways, it could have also been due to inhibition of carboxypeptidase M in the vagus nerve, where it is present in high concentration (Nagae *et al.*, 1992).

Whether carboxypeptidase M in pulmonary type I cells has functions unrelated to its enzymatic activity is unknown. Nevertheless, aminopeptidase N on intestinal epithelial cells acts as a receptor for coronaviruses (Yeager *et al.*, 1992), leaving the possibility that carboxypeptidase M also may be a receptor for infectious agents in the lungs. This hypothesis is supported by the recent discovery that a hepatitis B-virus-binding protein is a B-type carboxypeptidase with significant sequence identity to carboxypeptidases M, N and E (Kuroki *et al.*, 1995). Finally, the functions of carboxypeptidase M in many other locations remain to be explored. For example, in the placenta it may protect the fetus from maternally derived peptides. Its location in central nervous system (CNS) myelin and Schwann cells in peripheral nerves is intriguing, and may indicate a role for the growth or protection of neurons.

4.1.4 Carboxypeptidase U

A recent addition to this class of enzymes is an unstable blood-borne carboxypeptidase that is activated during coagulation. In 1989, several groups published that human serum has a higher (about 2–3-fold) arginine-carboxypeptidase activity than plasma, and that the difference could not be explained by changes in carboxypeptidase N activity (Campbell and Okada, 1989; Hendriks *et al.*, 1989; Sheikh and Kaplan, 1989). Interestingly, Erdös and colleagues (1964) noted that serum has a 9% higher carboxypeptidase activity than plasma, but the reason for the difference was not investigated. In retrospect, the reason for the smaller difference than in recent studies was the result of their use of hippuryl-Lys as the substrate, which is cleaved slower by this enzyme compared with carboxypeptidase N, and the addition of cobalt to the assay, which would inhibit carboxypeptidase U (Hendriks *et al.*, 1989; Tan and Eaton, 1995). Further investigations showed that the enzyme is a unique carboxypeptidase (Campbell and Okada, 1989; Hendriks *et al.*, 1990, 1992). In unrelated studies, a plasminogen-binding protein was fortuitously isolated and its sequence had significant homology to the pancreatic carboxypeptidases (Eaton *et al.*, 1991). Although many of the properties of this enzyme are quite similar to those of carboxypeptidase U, Eaton and coworkers believed the two enzymes were different and named the one they isolated "plasma carboxypeptidase B". However, based on similarities in properties and partial sequence information, investigators now believe they are identical (Shinohara *et al.*, 1994; Wang *et al.*, 1994). For the purposes of this review, they will be considered to be the same and the name, carboxypeptidase U, is used to avoid confusion. The same protein (as revealed by N-terminal sequencing) was recently isolated and named "thrombin-activatable fibrinolysis inhibitor (TAFI)" (Bajzar *et al.*, 1995).

4.1.4.1 Physical and Structural Properties

In the initial report, the size of the partially purified carboxypeptidase U was 435 kDa (Hendriks *et al.*, 1990). Complete purification on a plasminogen affinity column yielded a 60 kDa protein (Eaton *et al.*, 1991). This turned out to be the proenzyme, which, when activated by trypsin, yielded an active protein of 35 kDa (Eaton *et al.*, 1991). The size of the enzyme initially reported by Hendriks *et al.* (1990) may, therefore, represent carboxypeptidase U bound to plasminogen and possibly other proteins in a multimeric complex (Wang *et al.*, 1994). Indeed, treatment of the crude high molecular weight complex with 3 M guanidine, followed by chromatography, resulted in the isolation of an active enzyme with a major protein band at 53 kDa (Wang *et al.*, 1994). Based on the cDNA sequence, N-terminal sequencing after activation and similarities with the pancreatic carboxypeptidases, the active form has a molecular weight of 35 kDa (Eaton *et al.*, 1991). Further work is required to resolve this discrepancy. Although procarboxypeptidase U is a glycoprotein as shown by a reduction in mass from 60 kDa to 45 kDa after enzymatic deglycosylation (Eaton *et al.*, 1991), the four potential Asn-linked glycosylation sites in the sequence are all in the propeptide segment (Eaton *et al.*, 1991).

The reason why carboxypeptidase U is unstable in serum or after partial purification is not fully understood, although it is likely to involve proteolysis by enzymes activated during coagulation. The fact that the $t_{1/2}$ of carboxypeptidase U in its high molecular weight complex was only 15 min, whereas after guanidine treatment and chromatography to isolate the active enzyme (and presumably to remove any associated proteases), the $t_{1/2}$ increased to 55 min (Wang *et al.*, 1994), supports this hypothesis. In addition, the purified proenzyme can be activated by trypsin, but further incubation leads to an additional cleavage, reducing the size of the activated carboxypeptidase from 35 to 25 kDa, which results in inactivation (Eaton *et al.*, 1991). This is in contrast to the finding with carboxypeptidase B where a similar reduction in size does not inactivate the human pancreatic enzyme (Marinkovic *et al.*, 1977). When the activation with trypsin is carried out in the presence of the competitive inhibitor ϵ-amino caproic acid, the secondary cleavage is blocked without affecting hydrolysis of the propeptide, leading to production of a stable and fully active enzyme (Tan and Eaton, 1995).

4.1.4.2 *Enzymatic Properties*

Carboxypeptidase U does not have a marked preference for either C-terminal Arg or Lys or a penultimate Ala over Gly in short synthetic substrates (Hendriks *et al.*, 1989, 1990, 1992; Eaton *et al.*, 1991; Wang *et al.*, 1994; Tan and Eaton, 1995). However, with larger naturally occurring peptides, the enzyme cleaves C-terminal Arg faster than Lys (Tan and Eaton, 1995). The very low specificity constants (k_{cat}/K_m) of the substrates with carboxypeptidase U are due to the very high K_m values (63–220 mM; Tan and Eaton, 1995), which are about 1,000-fold higher than with carboxypeptidases M or N (Skidgel *et al.*, 1984b, 1989). Carboxypeptidase U has a pH optimum in the neutral range and in contrast to other metallocarboxypeptidases, Co^{2+} inhibits and Cd^{2+} activates the peptidase activity of the enzyme (Hendriks *et al.*, 1989; Tan and Eaton, 1995).

4.1.4.3 *Localization*

Carboxypeptidase U has only been found in blood, where it exists as an inactive proenzyme, which is activated during coagulation, presumably by a serine protease converted from a proenzyme during clotting (Campbell and Okada, 1989; Hendriks *et al.*, 1989; Eaton *et al.*, 1991). Although thrombin (Bajzar *et al.*, 1995) and plasminogen (Wang *et al.*, 1994) activate procarboxypeptidase U, it remains to be determined whether these are the major activation pathways *in vivo*, or if the propeptide can be cleaved by other serine proteases. The level of procarboxypeptidase U in plasma is high, an estimated 2–5 μg/ml (Eaton *et al.*, 1991) and after complete activation, around 10^{-7} M. As with other plasma proteins, procarboxypeptidase U is probably also synthesized in the liver, consistent with the cloning of its cDNA from a human liver library (Eaton *et al.*, 1991).

4.1.4.4 *Role of Carboxypeptidase U as a Kininase*

Because it was discovered relatively recently, the functions of carboxypeptidase U are still being explored. However, it likely plays an important role in the fibrinolytic pathway by regulating lysine-mediated plasminogen binding to proteins and cells, for example, by cleaving C-terminal Lys residues from α_2-antiplasmin, histidine-rich glycoprotein, fibrin, annexin II or α-enolase (Redlitz *et al.*, 1995).

With regard to its function as a kininase, it is of interest that one of the first groups to identify the enzyme used BK as a substrate (Sheikh and Kaplan, 1989). They noted that the conversion of BK to desArg^9-BK was much faster in serum than in plasma, being five-fold higher than could be accounted for by carboxypeptidase N (Sheikh and Kaplan, 1989). More recently, another group showed that, after activation of procarboxypeptidase U with trypsin in plasma, the enzyme removed the C-terminal Arg from BK (Shinohara *et al.*, 1994; Fig. 7.1). Finally, kinetics of BK hydrolysis were determined for the purified enzyme after trypsin activation (Tan and Eaton, 1995). Of all synthetic and naturally occurring substrates tested, BK had the lowest K_m and highest k_{cat}/K_m. However, the reported K_m is extraordinarily high (10 mM; Table 7.1), even though it is lower than that of all other substrates (K_m = 63–290 mM). For example, the K_m for BK with other peptidases ranges from about 0.18 to 520 μM (Table 7.1). This raises the question of the physiological relevance of carboxypeptidase U in degrading BK, which would normally be present at concentrations many orders of magnitude lower than the K_m. Because serum degrades BK five-fold faster than can be accounted for by the carboxypeptidase N level, carboxypeptidase U may be a significant kininase under certain circumstances. The apparent discrepancy between the observed BK degradation in serum and the calculated kinetic constants (Table 7.1) is not understood. Moreover, carboxypeptidase U would not cleave BK in blood because it is completely inactive as a proenzyme in plasma, but it may be activated if, for example, injury initiates the coagulation cascade.

4.2 SERINE CARBOXYPEPTIDASES

The involvement of serine carboxypeptidases in degrading BK *in vivo* has not been explored in detail. There are two lysosomal serine carboxypeptidases that can potentially affect kinin activity in pathological conditions, and these are prolylcarboxypeptidase and deamidase (cathepsin A/lysosomal protective protein). The active sites of serine carboxypeptidases contain a catalytic triad of amino acids characteristic of serine proteases. The order of the residues in the primary sequence (Ser, Asp, His) is unique and is the same as in the prolylendopeptidase (or prolyloligopeptidase) family of serine proteases (Tan *et al.*, 1993). Whereas the deamidase clearly belongs to the serine carboxypeptidase family with regard to sequence identity, prolylcarboxypeptidase has the sequence motifs characteristic of both the serine carboxypeptidases and the prolylendopeptidases, perhaps an indication that prolylcarboxypeptidase is a link between these two families of enzymes (Tan *et al.*, 1993).

4.2.1 Physical and Structural Properties

Prolylcarboxypeptidase (Yang *et al.*, 1968, 1970a) is a soluble single-chain protein of 58 kDa in sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and elutes as a symmetrical peak of 110 kDa in gel-filtration chromatography, indicating a dimer in its native form (Odya *et al.*, 1978; Tan *et al.*, 1993). It is a glycoprotein that contains 12% carbohydrate by weight, consistent with the presence of six potential Asn-linked glycosylation sites in the mature protein sequence (Tan *et al.*, 1993). The cDNA sequence indicates that the

protein contains a 30-residue signal peptide and 15-amino-acid propeptide (Tan *et al.*, 1993).

Deamidase was purified to homogeneity from platelets as a substance P-inactivating peptidase (Jackman *et al.*, 1990). In gel filtration, the enzyme has a molecular mass of 94 kDa whereas, in nonreducing polyacrylamide gel electrophoresis, it is 52 kDa indicating it exists as a homodimer. After reduction on SDS-PAGE, the 52 kDa protein dissociates into two chains of 33 and 21 kDa. The 33 kDa chain was labeled with [^{3}H]-diisopropylfluorophosphate, indicating it contains the active site serine residue (Jackman *et al.*, 1990). When the first 25 residues of each chain were sequenced, they were found to be identical with the sequences of the two chains of lysosomal protective protein, so named because it binds and maintains the activity and stability of β-galactosidase and neuraminidase in lysosomes (Galjart *et al.*, 1988). A defect in this protein is the cause of a severe genetic disease called galactosialidosis (d'Azzo *et al.*, 1982). Owing to its binding ability, deamidase can also be isolated from lysosomes in a high molecular weight active complex (500–600 kDa) (van der Horst *et al.*, 1989; Potier *et al.*, 1990).

4.2.2 Enzymatic Properties

Similar to other members of the serine protease family, prolylcarboxypeptidase and deamidase are inhibited by some compounds that react with the active site serine residue. For example, [^{3}H]-diisopropylfluorophosphate covalently labels the active site serine in both enzymes (Jackman *et al.*, 1990; Tan *et al.*, 1993). However, they are not inhibited by naturally occurring serine protease inhibitors such as aprotinin, soybean trypsin inhibitor, α_1-antitrypsin, α_1-antichymotrypsin, etc. (Odya *et al.*, 1978; Jackman *et al.*, 1990; Tan *et al.*, 1993). Because they are exopeptidases, they cannot bind to or cleave the internal peptide bonds in these inhibitors that are recognized by other serine proteases. Deamidase is inhibited by inhibitors of chymotrypsin-type enzymes such as Cbz-Gly-Leu-Phe-$CHCl_2$ and chymostatin due to its preference to cleave substrates with penultimate hydrophobic residues (Jackman *et al.*, 1990).

Both prolylcarboxypeptidase and deamidase have acidic pH optima (approximately 5.0) when hydrolyzing short synthetic peptide substrates (Jackman *et al.*, 1990; Tan *et al.*, 1993). Interestingly, with many longer substrates, both enzymes retain significant activity in the neutral range. For example, at pH 7.0, prolylcarboxypeptidase cleaves angiotensin II at 63% of the rate observed at pH 5 and deamidase cleaves BK at 72% of the rate at pH 5.5 (Jackman *et al.*, 1990; Tan *et al.*, 1993). In addition, the deamidation of C-terminally amidated peptides (such as -Met^{11}-NH_2 in substance P) by deamidase is optimal at neutral pH (Jackman *et al.*, 1990). As its name implies, prolylcarboxypeptidase cleaves peptides only if the penultimate residue is proline (Odya *et al.*, 1978; Tan *et al.*, 1993).

Deamidase/cathepsin A deamidates peptides such as substance P, neurokinin A and enkephalinamides, but also cleaves peptides with free carboxy-termini by carboxypeptidase action. It prefers peptides that contain hydrophobic residues in the P_1' and/or P_1 position. For example, it readily cleaves substance P free acid, converts angiotensin I to II (Jackman *et al.*, 1990), cleaves the chemotactic peptide fMet-Leu-Phe (Jackman *et al.*, 1995) and is the most potent endothelin degrading enzyme that has been identified (Jackman *et al.*, 1992, 1993).

4.2.3 Localization

Deamidase and prolylcarboxypeptidase are localized in lysosomes but, after release from cells by stimulation (e.g., platelets, white blood cells), they appear in the extracellular medium or biological fluids. For example, deamidase and prolylcarboxypeptidase were found in urine (Yang *et al.*, 1970a; Miller *et al.*, 1991) and prolylcarboxypeptidase was released into synovial fluid (Kumamoto *et al.*, 1981). In addition, lysosomal enzymes sometimes appear on the plasma membrane after exocytosis where they may be bound to other transmembrane or membrane-associated proteins (Skidgel *et al.*, 1991b).

Both enzymes are distributed ubiquitously and mRNA expression is highest in human placenta, lung and liver for prolylcarboxypeptidase (Tan *et al.*, 1993), and mouse kidney and placenta for deamidase/protective protein (Galjart *et al.*, 1990). Deamidase is also highly active in macrophages as well as in platelets, endothelial cells and fibroblasts (Jackman *et al.*, 1990, 1992, 1993, 1995). Prolylcarboxypeptidase is also found in white blood cells and fibroblasts, and is expressed at high levels in endothelial cells (Kumamoto *et al.*, 1981; Skidgel *et al.*, 1981).

4.2.4 Role of Serine Carboxypeptidases as Kininases

Because of their localization in lysosomes, serine carboxypeptidases would not normally have access to extracellular kinins. Since the kinin system is upregulated in inflammatory conditions where lysosomal enzymes can be released from leukocytes and other cells, these enzymes may then gain access to extracellular kinins. In addition, lysosomal serine carboxypeptidases could modulate kinin receptor signal transduction by cleaving kinins after ligand-mediated receptor endocytosis (Erdös *et al.*, 1989). This usually involves fusion of the endosomes with lysosomes and results in peptide degradation and receptor recycling (Yamashiro and Maxfield, 1988).

Because the best substrates of deamidase contain C-terminal hydrophobic residues, (e.g., endothelin 1, fMet-Leu-Phe, furylacryloyl-Phe-Phe, angiotensin I) (Jackman *et al.*, 1990, 1992, 1995), it is surprising that BK, with a C-terminal Arg, is a good substrate (Jackman *et al.*,

1990; Fig. 7.1). It is now clear, however, that the presence of a hydrophobic amino acid in the penultimate position allows deamidase to remove nonhydrophobic amino acids by its carboxypeptidase action. Thus, BK (-Phe-Arg) and angiotensin$_{1-9}$ (-Phe-His) are collectively cleaved by the enzyme (Jackman *et al.*, 1990).

Prolylcarboxypeptidase, on the other hand, cannot cleave native BK because it lacks a penultimate Pro residue. The ligand for the B_1 receptor, desArg^9-BK, has a C-terminal -Pro-Phe, a sequence that is readily cleaved by the enzyme (Yang *et al.*, 1968, 1970a; Odya *et al.*, 1978; Fig. 7.2). Thus, the carboxypeptidases potentially may be important regulators of the B_1 receptor signaling system. Metallocarboxypeptidases, such as carboxypeptidase M or N, or deamidase can generate desArg^9-BK (Fig. 7.1), which binds to B_1 receptors, upregulated in inflammation (see Chapter 8). Prolylcarboxypeptidase released from inflammatory cells would then cleave the C-terminal Phe from desArg^9-BK and inactivate it (Fig. 7.2). Previous studies reported that prolylcarboxypeptidase is released into the blood during endotoxin shock (Sorrells and Erdös, 1972), a potent stimulus for the upregulation of B_1 receptors (see above).

5. *Hydrolysis of Bradykinin by Aminopeptidases*

Aminopeptidases catalyze the removal of one amino acid at a time from the N-terminus of peptides and proteins. Although the N-terminal sequence of BK contains Pro in the second position, rendering the nonapeptide resistant to most aminopeptidases, aminopeptidase P specifically cleaves peptides with a Pro in the second position and, therefore, is capable of inactivating BK (Fig. 7.1). Aminopeptidases can also participate in the release of BK. For example, Lys-BK (kallidin) is liberated by tissue kallikrein, whereas BK is the product of plasma kallikrein (Bhoola *et al.*, 1992). Lys-BK is converted to BK by aminopeptidase action in the blood and similar activity has been detected in a variety of tissues (Erdös, 1979). However, as mentioned elsewhere in this chapter, it is not necessary for Lys-BK to be converted to BK before it is inactivated by peptidases; it can be inactivated directly by enzymes such as ACE and carboxypeptidase N (Erdös, 1979).

Dipeptidyl aminopeptidase IV specifically catalyzes the removal of dipeptides from the N-termini of peptides with a Pro in the second position (McDonald and Barrett, 1986), but it cannot cleave BK (Arg^1-Pro^2-Pro^3-...) because it does not hydrolyze Pro-Pro bonds. Nevertheless, as a secondary metabolic step it would release Pro-Pro from desArg^1-BK. Pro-Pro has been detected as one of the metabolites of BK after passing through the lung (Ryan *et al.*, 1968).

5.1 AMINOPEPTIDASE P

It has long been known that aminopeptidase P contributes to BK metabolism *in vitro* (Erdös *et al.*, 1963; Erdös and Yang, 1966) and in perfused rat lungs *in situ* (Ryan *et al.*, 1968). The enzyme was partially purified and characterized from pig kidney (Dehm and Nordwig, 1970), but was purified to homogeneity and its properties described in detail only recently (Hooper *et al.*, 1990; Simmons and Orawski, 1992; Orawski and Simmons, 1995; Vergas Romero *et al.*, 1995).

5.1.1 Physical and Structural Properties

Aminopeptidase P is membrane bound via a GPI anchor and can be solubilized with PI-PLC (Hooper and Turner, 1988; Hooper *et al.*, 1990; Simmons and Orawski, 1992; Orawski and Simmons, 1995). Under denaturing conditions in SDS-PAGE, aminopeptidase P runs as a single-chain protein with an M_r = 90,000–95,000, and is a glycoprotein containing about 17–25% carbohydrate by weight (Hooper *et al.*, 1990; Simmons and Orawski, 1992; Orawski and Simmons, 1995). After gel-filtration chromatography, the purified enzyme has a multimeric structure with a molecular mass of 220–360 kDa (Hooper *et al.*, 1990; Simmons and Orawski, 1992; Orawski and Simmons, 1995), which varies depending on the salt concentration (Orawski and Simmons, 1995). Partial protein sequencing of aminopeptidase P, purified from guinea-pig lung and kidney (Denslow *et al.*, 1994), and the full sequence of the pig kidney enzyme (Vergas Romero *et al.*, 1995) show that aminopeptidase P has some sequence similarity to human and *E. coli* prolidase as well as *E. coli* aminopeptidase P. Thus, these enzymes may constitute a newly recognized family of proline peptidases.

Early studies indicated the presence of a soluble (cytosolic) form of aminopeptidase P in kidney extracts (Dehm and Nordwig, 1970) and this form of the enzyme has been purified and characterized from other sources, including human lung, human erythrocytes, rat brain and human platelets (Sidorowicz *et al.*, 1984a,b; Harbeck and Mentlein, 1991; Vanhoof *et al.*, 1992). The soluble enzyme differs from the membrane-bound form both in structure and substrate specificity (see below). For example, the soluble aminopeptidase P purified from rat brain has an M_r = 71,000 in SDS-PAGE and a native M_r = 143,000 in gel filtration (Harbeck and Mentlein, 1991).

5.1.2 Enzymatic Properties

Membrane-bound aminopeptidase P cleaves peptides such as BK and neuropeptide Y (NPY), containing Pro in the second position, with a pH optimum in the neutral range (Orawski and Simmons, 1995; Simmons and Orawski, 1992). The tripeptide Gly-Pro-Hyp has been commonly used as a substrate to measure its activity, although the N-terminal tripeptide of BK (Arg-Pro-Pro)

is cleaved much faster (Simmons and Orawski, 1992; Orawski and Simmons, 1995). Aminopeptidase P also cleaves longer substrates, but not dipeptides such as Arg-Pro (Simmons and Orawski, 1992; Orawski and Simmons, 1995). Aminopeptidase P is a zinc metallopeptidase containing one zinc per mole of enzyme (Hooper *et al.*, 1990). The enzyme can be activated in the presence of Mn^{2+} with some substrates and is inhibited by chelating agents. Other inhibitors include sulfhydryl compounds, such as 2-mercaptoethanol, as well as sulfhydryl-reactive reagents, e.g., *p*-chloromercuriphenylsulfonate (Hooper *et al.*, 1990; Simmons and Orawski, 1992; Orawski and Simmons, 1995). Interestingly, many ACE inhibitors also inhibit aminopeptidase P, although generally with a K_i in the micromolar range (Hooper *et al.*, 1992) and Mn^{2+} can enhance their inhibitory effect (Orawski and Simmons, 1995). The inhibition is likely to be due to the presence of Pro or Pro-like structures in the ACE inhibitors, and the effective zinc binding moieties, which would chelate the active site metal of aminopeptidase P as they do in ACE. Whether some of the effects or side effects of ACE inhibitors could be due to inhibition of aminopeptidase P is not known.

The enzymatic properties of the soluble form of aminopeptidase P are very similar to those of the membrane-bound form with regard to pH optimum, inhibitors and activation by Mn^{2+} (Sidorowicz *et al.*, 1984a,b; Harbeck and Mentlein, 1991; Vanhoof *et al.*, 1992). Unlike the membrane-bound enzyme, however, the soluble form readily cleaves NH_2-X-Pro dipeptides (Sidorowicz *et al.*, 1984a,b; Harbeck and Mentlein, 1991; Simmons and Orawski, 1992; Vanhoof *et al.*, 1992; Orawski and Simmons, 1995). This may indicate that the soluble form is a different gene product and not simply the solubilized membrane-bound aminopeptidase P.

5.1.3 Localization

Aminopeptidase P-type activity has been detected in a variety of tissues and, based on purification studies, it is clear that lung and kidney contain high concentrations of membrane-bound aminopeptidase P, whereas the soluble or cytosolic enzyme is also present in brain, erythrocytes and platelets (Dehm and Nordwig, 1970; Sidorowicz *et al.*, 1984a,b; Hooper *et al.*, 1990; Harbeck and Mentlein, 1991; Simmons and Orawski, 1992; Vanhoof *et al.*, 1992; Orawski and Simmons, 1995). In the lung, the membrane-bound form is likely localized on the surface of pulmonary vascular endothelial cells with access to circulating peptides such as BK (Ryan, 1989).

5.1.4 Role of Aminopeptidase P as a Kininase

In the first studies on BK metabolism, it was discovered that one mechanism of inactivation consists of the removal of the N-terminal Arg residue (Erdös *et al.*, 1963). This type of activity in human erythrocytes (Erdös *et al.*, 1963) and porcine kidney (Erdös and Yang, 1966) was initially attributed to a prolidase, but it is now recognized that prolidase only cleaves X-Pro dipeptides, and that aminopeptidase P is the major enzyme responsible for removing the N-terminal Arg^1 from BK (Fig. 7.1).

The overall role of aminopeptidase P in the degradation of BK *in vivo* has been investigated in detail only recently. Early studies in perfused rat lungs showed that one pathway for BK degradation involved the removal of the N-terminal Arg residue (Ryan *et al.*, 1968). Two recent studies, also in perfused rat lungs, have carefully assessed the relative contributions of ACE and aminopeptidase P to BK metabolism. In both cases, it was concluded that ACE is the major kininase in rat lung, being responsible for about 70% of the total metabolism, and that essentially all of the BK hydrolysis could be accounted for by the combined actions of ACE and aminopeptidase P (Ryan *et al.*, 1994; Prechel *et al.*, 1995). Nevertheless, even when ACE activity was blocked completely, 75% of the BK was still inactivated during a single passage by aminopeptidase P alone (Ryan *et al.*, 1994; Prechel *et al.*, 1995). Studies *in vivo* corroborate these data where administration of the specific aminopeptidase P inhibitor, apstatin, doubled the hypotensive action of BK in rats (Scicli *et al.*, personal communication). Furthermore, apstatin was much less effective than lisinopril, a specific ACE inhibitor, confirming the primary role of ACE in kinin metabolism in rat lungs. The relevance of these findings is somewhat questionable because rat lungs contain extremely high levels of aminopeptidase P relative to other species (Ryan, 1989). For example, aminopeptidase P levels in rat lungs are 200-fold higher than in rabbit lungs, 30-fold higher than in pig lungs and 100-fold higher than in cat lungs (Ryan, 1989).

Aminopeptidase P could also play a role in the degradation of $desArg^9$-BK (Fig. 7.2), as the *in vitro* kinetics are essentially identical with those of BK (Simmons and Orawski, 1992; Orawski and Simmons, 1995). On the other hand, the specificity constant for $desArg^9$-BK with ACE is much less favorable than with BK owing to the much higher K_m (120–240 μM vs 0.18 μM for BK) (Inokuchi and Nagamatsu, 1981; Oshima *et al.*, 1985). Thus, ACE would probably play a much less prominent role in degrading $desArg^9$-BK *in vivo*, whereas the relative importance of aminopeptidase P would likely increase. However, the pathway for $desArg^9$-BK degradation *in vivo* has not yet been well characterized.

6. *Hydrolysis of Bradykinin by Other Endopeptidases*

Endopeptidases are enzymes that cleave a peptide in the interior of the peptide chain, with a specificity that depends mainly on the amino acids on one or both sides

of the peptide bond being cleaved. Endopeptidase 24.11 is such an enzyme, and its role as a kininase is discussed in Section 3 of the present review. Several other endopeptidases cleave BK, at least *in vitro*, (Fig. 7.1), but their roles as kininases *in vivo* are not fully understood. They are considered briefly below.

6.1 MEPRIN

Meprin was originally purified from a mouse kidney membrane fraction. It is the mouse homolog of human *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (PABA peptide) hydrolase and rat endopeptidase 2 (Beynon *et al.*, 1981; Butler *et al.*, 1987; Dumermuth *et al.*, 1991; Wolz and Bond, 1995). Purification and sequencing studies revealed meprin to consist of two unique but related subunits, α and β, and showed meprin to be a member of the astacin family of metalloproteases (Dumermuth *et al.*, 1991; Gorbea *et al.*, 1991; Jiang *et al.*, 1992; Johnson and Hersh, 1992; Wolz and Bond, 1995). The enzyme is an oligomeric, cell-surface protein, bound via the transmembrane β subunit to which the α subunits are either disulfide-linked or noncovalently bound (Gorbea *et al.*, 1991; Johnson and Hersh, 1994; Marchand *et al.*, 1994). Meprin has only been detected in kidney and intestine, and its expression varies from species to species, and even within different strains of the same species (Gorbea *et al.*, 1991; Jiang *et al.*, 1992, 1993). In contrast to endopeptidase 24.15, endopeptidase 24.11 and ACE, which hydrolyze only short peptides, meprin cleaves large protein substrates such as azocasein (Butler *et al.*, 1987). The α subunit of meprin hydrolyzes peptide and protein substrates longer than seven amino acids and cleaves the Gly-Phe bond of BK (Butler *et al.*, 1987; Fig. 7.1). Of all peptides tested, BK was hydrolyzed fastest among those cleaved at a single site (Wolz *et al.*, 1991; Table 7.1). This finding led to the synthesis of Phe^5(4-nitro)BK and the development of the first convenient spectrophotometric assay for meprin (Wolz and Bond, 1990, 1995). The β subunit, which is primarily in a latent form, does not cleave BK after activation (Kounnas *et al.*, 1991; Wolz and Bond, 1995). Meprin-α has a very broad substrate specificity and does not have strict requirements for residues adjacent to the cleavage site, but seems to prefer Pro in the P_2' or P_3' position (Wolz *et al.*, 1991). Meprin-α also cleaves α-melanocyte-stimulating hormone, neurotensin and LHRH, and it also hydrolyzes angiotensin I and II rather slowly (Wolz *et al.*, 1991). It is unlikely that meprin plays a dominant role in degrading kinins.

6.2 PROLYLENDOPEPTIDASE

Prolylendopeptidase is a cytoplasmic enzyme found in most tissues, with an especially high concentration in brain and kidney (Wilk, 1983). This serine protease has a molecular mass of about 70–77 kDa and is optimally active at pH 7.5 (Wilk, 1983). Cloning and sequencing of the porcine brain enzyme revealed that it differed from classical serine proteases in its sequence around the active site serine (Rennex *et al.*, 1991), and comparison with other known enzymes identified it as a member of a new family of serine proteases (Rawlings *et al.*, 1991). These enzymes have a catalytic triad similar to other serine proteases, but with a unique order of Ser... Asp... His in the primary sequence (Polgar, 1992).

Prolylendopeptidase cleaves at the C-terminal side of prolyl residues in peptides of about 30 or less amino acids (Wilk, 1983). It hydrolyzes BK by cleavage of the Pro^7-Phe^8 bond (Fig. 7.1), the same site as ACE and NEP (Wilk, 1983). With the purified porcine kidney enzyme, BK has a K_m of 7.5 μM and a V_{max} of 1.37 μmol min^{-1} mg^{-1} (Ward *et al.*, 1987; Table 7.1). Prolylendopeptidase can also cleave desArg^9-BK, releasing the C-terminal Phe (Fig. 7.2), with kinetics similar to those of BK (Ward *et al.*, 1987). As a consequence, the enzyme can inactivate ligands for B_1 and B_2 kinin receptors. Prolylendopeptidase cleaves a variety of other peptides containing Pro (Wilk, 1983).

The role of prolylendopeptidase is not well understood. Because of its localization in the cytosol, it would not normally have access to peptide hormones synthesized or secreted extracellularly and, even if it were present at significant concentrations in blood, its importance to BK metabolism would be minor. Experiments utilizing a potent prolylendopeptidase inhibitor, Cbz-Pro-prolinal, may help to elucidate its role in peptide metabolism (Wilk and Orlowski, 1983). The interpretation of the results with this inhibitor may be complicated by the fact that it is also a relatively potent inhibitor of the activity of prolylcarboxypeptidase, (Tan *et al.*, 1993), which cleaves some of the same substrates.

6.3 ENDOPEPTIDASE 24.15

Endopeptidase 24.15 was originally purified from the soluble fraction of rat brain homogenates and the enzyme from the extracts cleaved a variety of biologically active peptides (Orlowski *et al.*, 1983). Subsequent studies led to the realization that this enzyme is identical with two other enzymes that had been described and purified earlier: Pz-peptidase, which cleaves a synthetic collagenase substrate; and endo-oligopeptidase A, which was first described as a kininase (for reviews, see Tisljar, 1993; Barrett *et al.*, 1995).

Endopeptidase 24.15 has a pH optimum in the neutral range and is a metalloenzyme, as revealed by biochemical studies as well as its primary sequence, which contains the consensus HEXXH motif of zinc metalloenzymes (Orlowski *et al.*, 1983, 1989; Pierotti *et al.*, 1990; Tisljar, 1993). The enzyme is also sensitive to inhibition by sulfhydryl-reactive agents and is most stable and active in the presence of low concentrations of thiol-containing

compounds (Orlowski *et al.*, 1983, 1989; Tisljar and Barrett, 1990). This led one group to classify it as a cysteine peptidase and another to propose renaming it "thimet oligopeptidase" to describe this dual nature (Tisljar, 1993; Barrett *et al.*, 1995). However, the enzyme clearly is a metallopeptidase and its sensitivity to thiols is likely to result from the presence of a free cysteine removed five residues from the catalytic center, but this residue is probably not involved in the catalytic mechanism (Pierotti *et al.*, 1990).

Endopeptidase 24.15 is a single-chain enzyme of 645 amino acids with a molecular mass of 73 kDa (Pierotti *et al.*, 1990; Tisljar, 1993). Although it contains a single potential glycosylation site, there is no evidence that this enzyme is glycosylated (Pierotti *et al.*, 1990). Endopeptidase 24.15 is primarily a cytosolic enzyme, an observation that is consistent with the lack of a signal peptide (Pierotti *et al.*, 1990), although up to 20% of the enzyme may be membrane-associated (Acker *et al.*, 1987). How endopeptidase 24.15 is attached to membrane is not clear, as its primary sequence has no obvious transmembrane domain (Pierotti *et al.*, 1990). The enzyme is ubiquitously distributed, being especially highly concentrated in brain and testes (Tisljar, 1993; Barrett *et al.*, 1995).

Endopeptidase 24.15 has a rather broad substrate specificity, but is most active in cleaving at the carboxyl side of hydrophobic aromatic amino acids, especially those that contain an aromatic residue at the P'_3 position (Orlowski *et al.*, 1983). This explains why it readily cleaves BK at the Phe^5–Ser^6 bond with the aromatic Phe^8 residue in the P'_3 position (Orlowski *et al.*, 1983; Fig. 7.1, Table 7.1). Endopeptidase 24.15 hydrolyzes other biologically active peptides including neurotensin, substance P and LHRH, and converts large opioid peptides to enkephalins (Orlowski *et al.*, 1983; Chu and Orlowski, 1985). As with ACE and endopeptidase 24.11, endopeptidase 24.15 only hydrolyzes peptides shorter than about 20 residues (Chu and Orlowski, 1985; Tisljar, 1993).

The cytosolic localization of endopeptidase 24.15 makes its physiological relevance as a kininase questionable. Although a small portion (around 20%) is membrane bound in subcellular fractions of rat brain (Acker *et al.*, 1987), it is not clear whether this represents enzyme present on the exterior surface of the plasma membrane or bound to intracellular membranes. Nevertheless, some studies using the endopeptidase 24.15 inhibitor *N*-[1(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-pAB (cFP-AAF-pAB) (Orlowski *et al.*, 1988) suggested the possible involvement of the enzyme in inactivating BK *in vivo* or *in situ*. For example, cFP-AAF-pAB enhanced BK-induced contractions of rat uterus (Schriefer and Molineaux, 1993) and blocked most BK degradation by rat hypothalamic slices (McDermott *et al.*, 1987). Intravenous infusion of cFP-AAF-pAB to normotensive rats resulted in an immediate drop in blood pressure of up to 50 mm Hg that was blocked by a B_2 receptor antagonist (Genden and Molineaux, 1991). The inhibitor also increased the potency of an i.v. infusion of BK by ten-fold. However, these data were recently shown to be due primarily to indirect inhibition of ACE by cFP-AAF-pAB after hydrolysis of the inhibitor by endopeptidase 24.11 (Cardozo and Orlowski, 1993) as it had no effect in animals pre-treated with an ACE inhibitor (Yang *et al.*, 1994; Telford *et al.*, 1995).

6.4 ENDOPEPTIDASE 24.16

Endopeptidase 24.16 (neurotensin-degrading enzyme; neurolysin) was first described as an enzyme that degrades neurotensin in rat brain membranes (Checler *et al.*, 1983). Because its properties are similar to those of endopeptidase 24.15, it was not clear that it represented a new enzyme. However, subsequent purification and characterization of the enzyme proved that it is unique (Checler *et al.*, 1986, 1995; Millican *et al.*, 1991). Recent studies have revealed that the enzyme is likely to be identical with the soluble angiotensin II-binding protein and rabbit microsomal endopeptidase, and is at least partly localized in the mitochondrial intermembrane space (Barrett *et al.*, 1995; Serizawa *et al.*, 1995). Its sequence identifies it as a member of the same family as endopeptidase 24.15 (Barrett *et al.*, 1995; Serizawa *et al.*, 1995). Purified endopeptidase 24.16 cleaves BK at the same site as endopeptidase 24.15, (Fig. 7.1) (Millican *et al.*, 1991). The cytosolic and mitochondrial localization of endopeptidase 24.16 appears to preclude it from metabolizing BK under normal circumstances.

7. *Conclusions*

It is clear after decades of research that ACE on the vascular endothelial cell surface is the most important inactivator of blood-borne BK. However, it has also become evident that BK may act primarily in an autocrine and paracrine fashion (Carretero and Scicli, 1989), establishing the importance of local regulation of its activity by enzymes on cell surfaces (Skidgel, 1992). Thus, the assortment of other enzymes that can inactivate BK may be important in a variety of physiological and pathological situations. In addition, most physiological systems have redundant pathways of metabolism so that the abolishment of one pathway is compensated for by the presence of others. This is vividly demonstrated by the pharmacological inhibition of ACE in hypertension. Although some side effects might be attributed to potentiation of responses to BK (e.g., cough, angioedema), the drugs are surprisingly well tolerated, demonstrating the effectiveness of other peptidases in minimizing the potential deleterious effects of excess kinins. The cleavage of BK by other peptidases

at specific sites should lead to the future development of peptidase inhibitors that may be useful in conditions where potentiation of the actions of BK and elevation of its concentration are desirable features.

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