Human Kidney "Enkephalinase", a Neutral Metalloendopeptidase That Cleaves Active Peptides†

John T. Gafford, Randal A. Skidgel, Ervin G. Erdös,* and Louis B. Hersh

ABSTRACT: After extracting converting enzyme from a membrane fraction of homogenized human kidney, "enkephalinase" activity was solubilized with Triton X-100. Ion-exchange chromatography resolved two peaks of the "enkephalinase" activity, both of which cleaved Leu^5-enkephalin at the Gly^3–Phe^4 bond. The major "enkephalinase" form was purified 1140-fold to homogeneity with a 14% yield. This homogeneous "enkephalinase" had a specific activity of 46 μmol min^{-1} mg^{-1} with Leu^5-enkephalin as substrate. The purified enzyme, in addition to hydrolyzing Leu^5-enkephalin, cleaved synthetic substrates with protected N- and C-terminal ends. On the basis of the specificity of the enzyme and its inhibition by chelating agents, human "enkephalinase" can be classified as a neutral metalloendopeptidase with a broad substrate specificity. The activity of this neutral endopeptidase with several biologically active peptides was compared to that of homogeneous human kidney converting enzyme. Both enzymes inactivated bradykinin by release of the C-terminal dipeptide but were inhibited differentially by specific inhibitors. Comparison of hydrolysis of bradykinin with that of its protected C-terminal peptide indicated that the neutral endopeptidase is more active toward the larger substrate than is converting enzyme. Although the neutral endopeptidase did not convert angiotensin I to II, it did hydrolyze angiotensin I at Pro^7–Phe^8 and inactivate angiotensin II by cleavage at the Tyr^4–Ile^5 bond.

The metabolism of enkephalins has generated considerable interest because of their importance as opioid peptides and the simplicity of their structure. It was initially suggested that enkephalins are degraded by aminopeptidase action in which cleavage of the N-terminal tyrosine leads to a loss of biological activity (Pert et al., 1976; Hambrook et al., 1976; Dupont et al., 1977; Marks et al., 1977; Grynbaum et al., 1977; Craves et al., 1978; Lane et al., 1977). Subsequently, the cleavage of the Gly^3–Phe^4 bond by peptidyl dipeptidase, also called dipeptidyl carboxypeptidase (converting enzyme or kininase II; CE[1]), was described (Erdös et al., 1978). Indeed, of the biologically active peptides, homogeneous human CE hydrolyzed Met^5-enkephalin fastest (Stewart et al, 1981), although the K_m of the enkephalins with this enzyme was higher than that of angiotensin I or bradykinin. Schwartz and his associates (Malfroy et al., 1978) and Sullivan et al. (1978) described a second enzyme in rodent brain that also cleaves at the Gly^3–Phe^4 bond of the enkephalins, but has a much lower K_m than CE. The K_m for this enzyme was initially estimated to be in the nanomolar range (Malfroy et al., 1978), but in more recent studies, the K_m was $\sim$70 μM (Rush & Hersh, 1982). This enzyme, classified as a second dipeptidyl carboxypeptidase (Schwartz et al., 1981), was called the true membrane-bound "enkephalinase". "Enkephalinase" activity has been found in a variety of tissues (Llorens & Schwartz, 1981) and was partially purified from kidney (Mumford et al., 1981), pituitary (Almenoff et al., 1981; Orlowski & Wilk, 1981), and brain (Gorenstein & Snyder, 1979, 1980; Rush & Hersh, 1982; Arregui et al., 1979). The properties of "enkephalinase" resemble those of an endopeptidase originally found in the brush border of rabbit and pig kidney (Kerr & Kenny, 1974a,b.) and intestine (Danielsen et al., 1980) that cleaved the B chain of insulin at the amino side of leucine or phenylalanine. Recent studies suggest that "enkephalinase" is not a dipeptidyl carboxypeptidase but is indeed a neutral metalloprotease (Hersh, 1982; Almenoff et al., 1981; Mumford et al., 1981; Blumberg et al., 1981; Orlowski & Wilk, 1981; Fulcher et al., 1982) with a specificity similar to a group of bacterial neutral endopeptidases of which thermolysin is the best characterized. Thus, in this paper, we refer to "enkephalinase" as neutral metalloendopeptidase (NEP).

We report here the purification of human kidney NEP to homogeneity. This enzyme is a major active constituent of human kidney present in the membrane fraction after the extraction of CE and readily cleaves peptides such as enkephalins, bradykinin, and angiotensin II but does not convert angiotensin I to II. Human NEP cleaves the penultimate bond in enkephalins and bradykinin but it also hydrolyzes peptide bonds more distant from the C-terminal end; thus, it is not a peptidyl dipeptidase but an endopeptidase-type enzyme.

Experimental Procedures

Materials

[*tyrosyl*-^{3}H]-Leu^5-enkephalin was obtained from New England Nuclear Corp., Boston, MA. Leu^5-enkephalin was purchased from Vega Chemical Co., Tucson, AZ. Abz-Ala-Tyr-Leu-Ala-Gly-Nba was a generous gift from Dr. James Powers, Georgia Tech. Bz-Gly-His-Leu-, Bz-Gly-Phe-Arg, bradykinin, and angiotensin I and II were purchased from Bachem, Torrance, CA. The C-terminal hexa-, penta-, and tetrapeptide fragments of angiotensin II were donated by Dr. M. Bumpus of the Cleveland Clinic Foundation. Captopril [(S)-*N*-(3-mercapto-2-D-methylpropanoyl)proline] was donated by Z. Horovitz of Squibb Co., MK 421 [(*S*)-[*N*-(1-carboxy-3-phenylpropyl)alanyl]proline ethyl ester] by Dr. C. Sweet of Merck, Keto-ACE [(*S*)-(5-benzamido-4-oxo-6-phenylhexanoyl)proline] by Dr. R. Almquist of S.R.I., In-

† From the Departments of Pharmacology, Internal Medicine, and Biochemistry, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235. *Received December 27, 1982.* This work was supported in part by Grants HL 28813 HL 20594, and HL 16320 (National Institutes of Health), DA 02243 (National Institute of Drug Abuse), and I-391 (Robert A. Welch Foundation).

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Abz, 2-aminobenzoyl (2-NH_2–C_6H_4–CO–); Nba, 4-nitrobenzylamine (–N–CH_2–$C_6H_4NO_2$); Bz, α-*N*-benzoyl; MNA, 4-methoxy-2-naphthylamine; $NaDodSO_4$, sodium dodecyl sulfate; CE, angiotensin I converting enzyme; HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

0006-2960/83/0422-3265$01.50/0

ternational, Menlow Park, CA, and thiorphan [(*R*,*S*)-*N*-(3-mercapto-2-benzylpropanoyl)glycine] by Dr. J. Berger of Schering Corp.

Methods

Enzyme Assays. NEP activity was routinely measured by following the formation of [^{3}H]Tyr-Gly-Gly from [*tyrosyl*-^{3}H]-Leu^5-enkephalin. Reaction mixtures contained 50 mM Tris-HCl, pH 7.4, 0.1 mM [*tyrosyl*-^{3}H]-Leu^5-enkephalin (sp act. 7×10^7 cpm/μmol), 0.1 mM aminopeptidase inhibitor puromycin, 1 μM CE inhibitor captopril, and the enzyme in a final volume of 20 μL. After a 5–20-min incubation, the reaction was terminated by boiling and labeled Tyr-Gly-Gly was separated from Leu^5-enkephalin by thin-layer chromatography as previously described (Rush & Hersh, 1982). Unless stated, all kinetic studies with homogeneous NEP were performed in 0.1 M Mes buffer, pH 6.5, and 0.1% Triton X-100 in the absence of puromycin and captopril. The hydrolysis of Abz-Ala-Tyr-Leu-Ala-Gly-Nba was measured by following the increase in fluorescence that occurs when the fluorescent aminobenzyl group is liberated from the fluorescent-quenching nitrobenzylamine group by cleavage of a peptide bond (R. Rush, M. Mitas, T. Tanaka, J. Powers, and L. Hersh, unpublished results). Suc-Ala-Ala-Phe-MNA hydrolysis was measured in a two-step procedure as previously described (Orlowski & Wilk, 1981). In the first step, the substrate is cleaved by NEP at the Ala–Phe bond to release Phe-MNA as a product. After the reaction has been stopped, aminopeptidase M is added to hydrolyze Phe-MNA to free MNA, which is measured fluorometrically. Bz-Gly-His-Leu hydrolysis was assayed by coupling the His-Leu released with *o*-phthalaldehyde (Yang & Neff, 1972).

Measurement of Bz-Gly-Phe-Arg Hydrolysis. Bz-Gly-Phe-Arg hydrolysis was measured by detecting the release of Phe-Arg with fluorescamine (Udenfriend et al., 1972). The reaction mixture (0.5 mL) was diluted by the addition of 1 mL of 0.2 M potassium phosphate buffer, pH 8.0. After mixing, 0.5 mL of 0.25% (w/v) of fluorescamine in acetone was added. Formation of Phe-Arg was detected fluorometrically at an excitation wavelength of 390 nm and an emission wavelength of 480 nm. The instrument was standardized with Phe-Arg.

Thin-Layer Chromatography. Thin-layer chromatography was carried out on silica gel plates in 1-butanol/acetic acid/water (4/1/1). The spots were visualized with fluorescamine.

High-Pressure Liquid Chromatography. HPLC analyses were conducted in a Waters automated gradient system that consisted of an M-6000A pump, an M-45 pump, a WISP 710B automatic injector, a Model 720 system controller, an M730 data module, and a Model 441 absorbance detector. Peptide products were separated on a Waters μBondapak C_{18} reverse-phase column by using an increasing linear gradient of acetonitrile in 0.02% trifluoroacetic acid in H_2O and were detected at 214 nm. The column was equilibrated for 7 min at initial conditions between runs. Hydrolysis products of bradykinin and Leu^5-enkephalin were separated with a 15-min linear gradient from 0 to 60% acetonitrile in 0.02% trifluoroacetic acid/H_2O. The products of angiotensin II hydrolysis were separated with a linear gradient of 0–15% acetonitrile in 6 min followed by a 14-min linear gradient of 15–30% acetonitrile.

Bioassay. The inactivation of the spasmogenic action of bradykinin and angiotensin II was determined on the isolated rat uterus in estrus (Erdös & Sloane, 1962). The peptides in 2 μM concentration were incubated at 37 °C with enzyme, and aliquots were injected into the tissue bath at regular intervals. The buffer was 0.1 M Tris, pH 7.4; however, with CE, the buffer also contained 0.1 M NaCl.

$NaDodSO_4$ Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was done in 7.5% acrylamide gels (Laemmli, 1970). $NaDodSO_4$–polyacrylamide gel electrophoresis gels were stained for protein with Coomassie Blue and for carbohydrate with the periodic acid–Shiff's reagent of Kapitany & Zebrowski (1973).

Neutral Sugars. Total neutral sugars were determined by the phenol–sulfuric acid method (Dubois et al., 1956).

Purification of Human Kidney NEP. Human kidneys, 3–8 h post mortem, were obtained from the Department of Forensic Pathology, University of Texas Health Science Center, and stored at −20 °C for up to 2 months before use. Thawed tissue (500 g) was homogenized in 5 volumes of 0.02 M potassium phosphate buffer, pH 8.0, containing 0.25 M sucrose. The crude homogenate was treated as previously described for the purification of human kidney CE, using acid precipitation of the particulate fraction and trypsin to solubilize CE (Stewart et al., 1981; Weare et al., 1982).

The human CE solubilized by trypsin was purified to homogeneity by reverse immunoadsorption (Weare et al., 1982). The residual membrane fraction was devoid of CE activity but contained at least 50% of the estimated NEP activity, although it was difficult to obtain a measurement of NEP activity in the presence of high levels of CE, even with added CE inhibitors.

The residual membrane fraction was resuspended in 350 mL of 50 mM Tris-HCl buffer, pH 7.4. Triton X-100 was added to a final concentration of 1% and the solution stirred for 1 h at 37 °C to solubilize the enzyme. After centrifugation for 2 h at 100000*g*, the supernatant was applied to a DE-52 column (5 × 40 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 1% Triton X-100 (buffer A). After the column was washed with 5 volumes of buffer A, NEP activity was eluted batchwise with buffer A containing 25 mM sodium chloride. A linear salt gradient increasing from 25 to 600 mM sodium chloride in buffer A (5500 mL) was then applied to the column. A second peak of NEP activity eluted with 0.1 M sodium chloride and was not purified further.

The fractions containing the first peak of activity from the DE-52 column were concentrated in an Amicon hollow-fiber concentrator and dialyzed against two changes of 20 volumes of 50 mM Tris-HCl buffer, pH. 7.4, containing 0.1% Triton X-100 (buffer B). The dialyzed enzyme was further concentrated 2-fold in the hollow-fiber concentrator and then dialyzed against 40 volumes of 25 mM imidazole buffer, pH 7.4, containing 0.1% Triton X-100. The dialyzed enzyme was then subjected to chromatofocusing according to the method described in the Pharmacia Fine Chemical Co. bulletin. A 200-mL column was employed with a pH gradient from 7.0 to 4.0.

The active fractions from the chromatofocusing column were pooled and concentrated 20-fold in the hollow-fiber concentrator and then dialyzed against two changes of 40 volumes of buffer B and then against 40 volumes of 2 mM sodium phosphate buffer, pH 6.8, containing 0.1% Triton X-100. The dialyzed enzyme was applied to a column of hydroxylapatite (180 mL) equilibrated with the 2 mM sodium phosphate buffer. After the column was washed with 3 column volumes of starting buffer, a linear 2-L gradient increasing from 2 to 75 mM in sodium phosphate buffer (pH 6.9) containing 0.1% Triton X-100 was applied to the column. The enzyme eluted in a broad peak over the entire gradient; however, the majority

Table I: Purification of Human Kidney "Enkephalinase"

purification step	vol (mL)	protein (mg)	activity (units)[a]	sp act. (units/mg)	yield (%)	purification (x-fold)
homogenate	3150	85 680	3663[b]	0.04	(100)	(1.0)
membrane fraction	2625	21 525	1757[b]	0.08	48	2
membrane fraction after trypsin treatment	350	1 995[c]	902	0.45	25	11
Triton-solubilized enzyme	290	783	600	0.80	16	20
DE-52 ion exchange	210	116	572	4.9	15.7	122
chromatofocusing	36	45	540	12.0	14.7	300
hydroxylapatite	17	16.2	500	30.9	13.7	773
Sephadex G-200	12	11	502	45.6	13.7	1140

[a] 1 unit equals 1 μmol of Leu[5]-enkephalin hydrolyzed per min at 37 °C. [b] The presence of contaminating aminopeptidases and CE resulted in nonlinear assays of activity vs. enzyme concentration. The values listed are probably overestimates of the actual NEP activity due to the formation of Tyr-Gly-Gly by contaminating CE. [c] The membrane fraction was stored at −20 °C prior to Triton treatment.

of the enzyme activity appeared between 5 and 35 mM sodium phosphate buffer. The pooled hydroxylapatite fractions (1100 mL) were first concentrated to 200 mL in a hollow-fiber concentrator and then to 11 mL with an Amicon ultrafiltration apparatus with a YM-10 membrane. The concentrated fractions from hydroxylapatite chromatography were dialyzed against buffer B. Gel filtration of the enzyme on a column of Sephadex G-200 (95 × 2 cm) in buffer B yielded homogeneous enzyme (Table I). Protein was determined by the method of Wang & Smith (1975) with bovine serum albumin as a standard.

Results

Purification of Human Kidney NEP. Cleavage of enkephalins at the Gly[3]–Phe[4] bond is catalyzed by both NEP (commonly referred to as "enkephalinase") and CE. Since kidney is rich in CE, where it is concentrated on the brush border (Hall et al., 1976), we devised a method to separate these activities at an early stage of purification. CE, after precipitation of the membrane particles at pH 5, was released by trypsin treatment (Weare et al., 1982). The residual membranes contain NEP that is solubilized with Triton X-100. Chromatography of the solubilized material on DE-52 anion-exchange resin resolved two forms of NEP, A_1 and A_2 (Figure 1), as previously noted for the enzyme from rat brain (Gorenstein & Snyder, 1979, 1980; Rush & Hersh, 1982). Recovery of enzyme activity at this stage of purification was greater than 100%, presumably due to the removal of inhibitory factors. Most of the aminopeptidase activity that acts on Leu[5]-enkephalin was eluted with the A_2 peak. NEP activity in the first peak (A_1) was further purified by chromatofocusing, followed by hydroxylapatite chromatography and gel filtration on a Sephadex G-200 column. This procedure resulted in an 1140-fold purification of the enzyme with a 14% yield (Table I). The specific activity of the purified enzyme with Leu[5]-enkephalin as substrate is 46 μmol min^{-1} (mg of protein)$^{-1}$. The enzyme was homogeneous, as indicated by a single band in $NaDodSO_4$–polyacrylamide gel electrophoresis visualized by staining for either protein or carbohydrate (Figure 2). Thus, NEP is a glycoprotein with a minimal molecular weight, estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis of 90 000.

Kinetic Properties of Purified NEP. The optimum pH for hydrolysis of Leu[5]-enkephalin is 6.5 (Figure 3). The turnover number for Leu[5]-enkephalin hydrolysis is 4680 min^{-1} (Table II). In addition to hydrolyzing Leu[5]-enkephalin, the purified enzyme cleaved the synthetic substrates Suc-Ala-Ala↑Phe-MNA, Abz-Ala-Tyr↑Leu-Ala-Gly-Nba, and Bz-Gly↑Phe-Arg but not Bz-Gly-His-Leu (Table II). The synthetic substrates were hydrolyzed at the amino side of the hydrophobic amino acids leucine and phenylalanine. The potent NEP inhibitors

Table II: Hydrolysis of Leu[5]-enkephalin and Synthetic Substrates[a]

substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
Leu[5]-enkephalin	74	52	4680	63
Suc-Ala-Ala-Phe-MNA	14	25	2250	161
Abz-Ala-Tyr-Leu-Ala-Gly-Nba	100	8.6	774	7.7

[a] Enzyme assays were conducted as described under Methods. Substrate concentrations were generally varied over a 10-fold range. Kinetic constants were determined from double-reciprocal plots of velocity vs. substrate concentration.

Table III: Inhibition of Purified Human Renal NEP[a]

inhibitor	concn	inhibition (%)
phosphoramidon	1 × 10^{-7} M	96
thiorphan	1 × 10^{-7} M	42
NHOH-Bz-malonyl-Ala-Gly-NH_2[b]	5 × 10^{-7} M	33
NHOH-Bz-malonyl-Gly-Leu-NH_2[c]	5 × 10^{-7} M	26
EDTA	1 × 10^{-3} M	93
o-phenanthroline	1 × 10^{-3} M	97
dithiothreitol	1 × 10^{-3} M	81
dimercaptopropanol (BAL)	1 × 10^{-4} M	87
captopril	1 × 10^{-5} M	0
MK 421	1 × 10^{-6} M	0
Keto-Ace	1 × 10^{-5} M	0
puromycin	1 × 10^{-3} M	0
N-ethylmaleimide	1 × 10^{-3} M	0
aprotinin	100 units	10
antiserum to human CE	1/100 v/v	0

[a] NEP activity was measured with 50 μM Leu[5]-enkephalin as substrate as described under Methods. [b] Hydroxamate of benzylmalonylalanylglycinamide. [c] Hydroxamate of benzylmalonylglycylleucinamide.

phosphoramidon (Hudgin et al., 1981; Mumford et al., 1981) and thiorphan (Llorens et al., 1980) inhibited the hydrolysis of Leu[5]-enkephalin and the synthetic substrates at a concentration of 0.1 μM. In addition, similar to the rat brain enzyme, human renal NEP was inhibited by a variety of compounds (e.g., chelating agents and dithio compounds such as dimercaptopropanol or dithioerythritol; Table III). Specific inhibitors of CE such as captopril (Cushman & Ondetti, 1980), MK 421 (Gross et al., 1981), and Keto-ACE (Almquist et al., 1980) did not inhibit NEP activity at the concentrations tested.

Inactivation of Bradykinin. Initially, as measured by bioassay, NEP (0.3 μg/ml) inactivated 50% of the bradykinin (2 μM) in 7 min. This inactivation was blocked by phosphoramidon (0.1 μM) but not by captopril (1 μM). Conversely, CE, at approximately the same concentration as NEP, inactivated bradykinin at the same rate in bioassay as NEP,

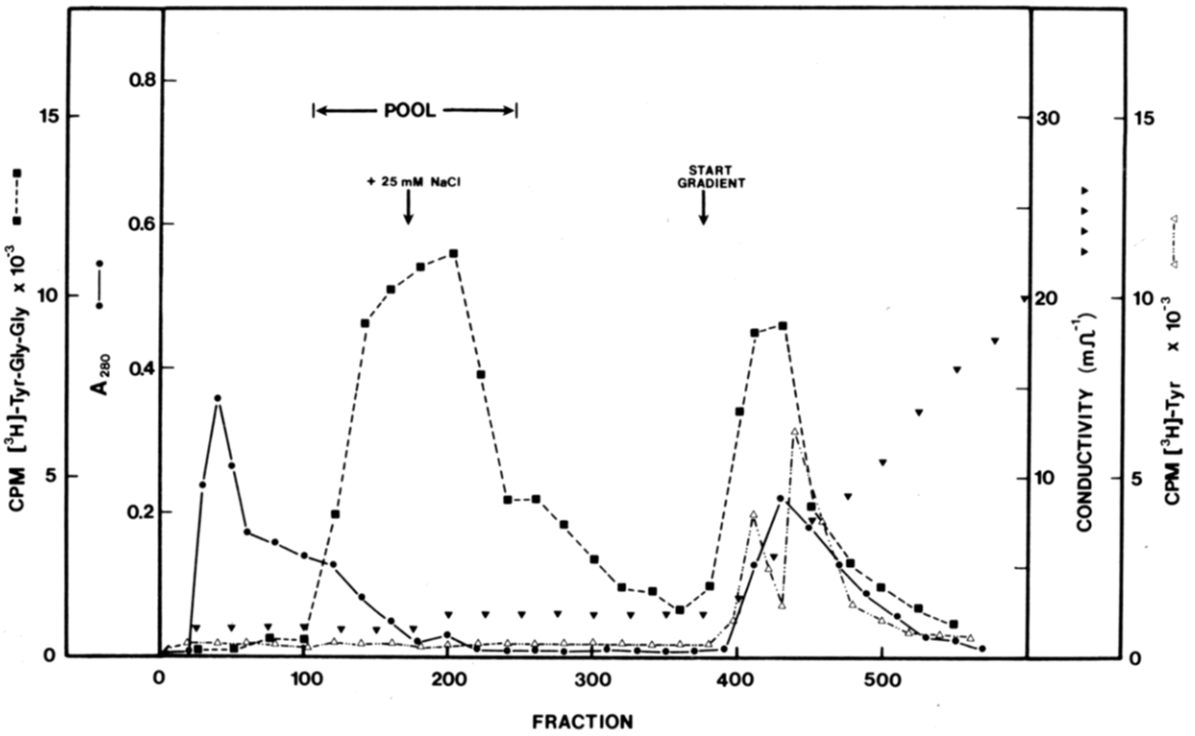

FIGURE 1: DEAE-cellulose chromatography of human renal NEP. The major peak of activity was eluted with 25 mM NaCl in 50 mM Tris-HCl, pH 7.4, containing 1% Triton X-100 and was called NEP A_1. A linear gradient of 25–600 mM NaCl in the same buffer eluted a second peak of activity (NEP A_2) that coeluted with two peaks of aminopeptidase activity (detected by the release of [^{3}H]Tyr from [*tyrosyl*-^{3}H]-Leu^5-enkephalin). Only NEP A_1 was purified further. For details, see Methods.

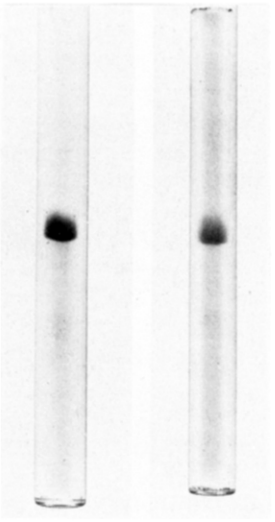

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified human renal NEP: (left gel) 20 μg of enzyme stained for protein with Coomassie Blue; (right gel) 40 μg of enzyme stained for carbohydrate with periodic acid–Schiff's reagent.

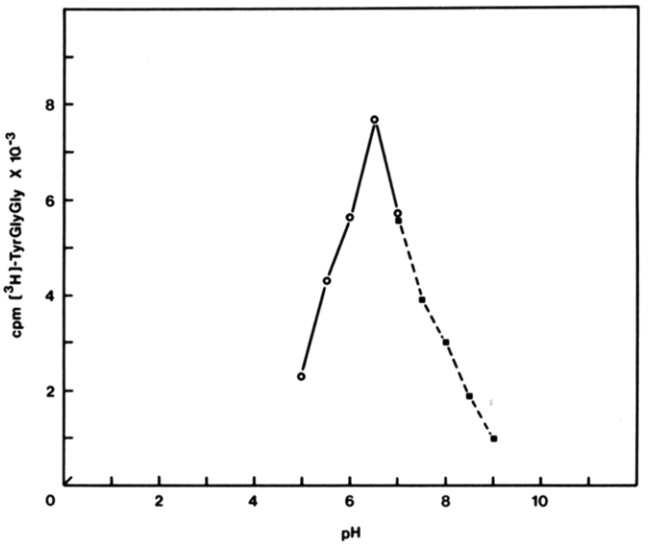

FIGURE 3: pH–activity profile for purified human renal NEP. [*tyrosyl*-^{3}H]-Leu^5-enkephalin hydrolysis was measured as described under Methods in the presence of 0.1 M Mes buffer (pH 5.0–7.0) or 0.1 M Tris-HCl buffer (pH 7.0–9.0).

but this reaction was inhibited by captopril and not by phosphoramidon.

The primary site of hydrolysis in bradykinin by NEP was shown to be at the Pro^7–Phe^8 bond. Thus, NEP, similar to kininase II, releases a C-terminal Phe^8–Arg^9. This was established by thin-layer chromatography on plates of silica gel and by HPLC. As in bioassay, the release was blocked with phosphoramidon but not by captopril.

The kinetics of hydrolysis of bradykinin by NEP was monitored by HPLC. During the early period of the reaction (less than 30 min), NEP cleaved bradykinin between the Pro^7–Phe^8 bond, releasing only Phe^8–Arg^9. However, at higher NEP concentrations and longer incubation times, additional peaks appeared on the HPLC tracings, indicating a secondary site of cleavage. Here, the 1–7 heptapeptide peak, which eluted at 12.8 min, decreased and was replaced by peaks that eluted at 9.0 and at 11.5 min. This presumably resulted from cleavage of the Gly^4–Phe^5 bond (the same bond cleaved in enkephalins) to yield the 1–4 tetrapeptide and 5–7 tripeptide. The K_m of bradykinin with NEP was 120 μM.

Table IV compares the kinetic parameters of the hydrolysis of bradykinin and its protected C-terminal dipeptide Bz-Gly-Phe-Arg by the two human kidney enzymes CE and NEP. Although bradykinin has a much lower K_m with CE, NEP has an ~10-fold higher turnover number. The protected C-terminal dipeptide fragment of bradykinin has about the same K_m with either enzyme, but its turnover number with CE is

Table IV: Kinetic Parameters of the Hydrolysis of Bradykinin and Bz-Gly-Phe-Arg by Human Renal Enzymes

	CE[a]			NEP		
substrates	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
bradykinin	1	500	500	120	4770	40
Bz-Gly-Phe-Arg	560	23 000	41	500	1656	3.3

[a] Stewart et al., 1981.

~15 times higher. Although CE exhibits a higher specificity (catalytic) constant with either substrate under saturating conditions CE, a dipeptidyl peptidase, cleaves the shorter substrates faster while the endopeptidase hydrolyzes the nonapeptide preferentially.

Hydrolysis of Angiotensins. The decapeptide angiotensin I is inactive on the isolated rat uterus, while angiotensin II, its octapeptide derivative, contracts it. We did not detect any conversion of angiotensin I to II by purified NEP. Angiotensin II, however, was inactivated by NEP. At 2 μM angiotensin II concentration, 0.75 μg of NEP inactivated 50% of the substrate in 18 min as measured by bioassay.

The site of cleavage of angiotensin II was determined by thin-layer chromatography and HPLC. In thin-layer chromatography, the spot due to angiotensin II (50 nmol) disappeared, and several new spots appeared after 20 min of incubation with 1.5 μg of NEP. The major spot migrated the same distance as the synthetic C-terminal tetrapeptide fragment of angiotensin II; thus, the first site of cleavage in angiotensin II by human NEP was identified as the Tyr^4-Ile^5 bond. Puromycin (1 mM) did not change the cleavage pattern, but phosphoramidon (1 μM) blocked the hydrolysis.

These findings were confirmed by HPLC. In these experiments, an initial cleavage of the Tyr^4–Ile^5 bond yielded the N- and C-terminal tetrapeptide fragments of angiotensin II. After prolonged incubation of NEP with angiotensin II, the 5–8 tetrapeptide peak, eluted at 16.4 min, remained intact, while the 1–4 tetrapeptide peak, which eluted at 12.5 min, decreased, giving rise to two new peaks eluting at 3.6 and 11.3 min. Since no free tryosine was liberated, these were presumed to be Asp^1–Arg^2 (3.6 min) and Val^3–Tyr^4 (11.3 min) resulting from the cleavage of the Arg^2–Val^3 bond (Figure 4).

From these experiments, it was calculated that angiotensin II was hydrolyzed by the purified human kidney NEP at a V_{max} of 50 μmol min^{-1} mg^{-1} and a K_m of 280 μM. While NEP did not convert angiotensin I to angiotensin II, it did readily hydrolyze angiotensin I to other products. The initial cleavage resulted in two major peaks resolved by HPLC that eluted at 15.5 and 17.3 min. The peak at 15.5 min coeluted with standard N-terminal heptapeptide (Asp^1–Pro^7) angiotensin I; thus, the initial cleavage was the the Pro^7–Phe^8 bond. Longer incubations at higher enzyme concentrations resulted in cleavage patterns similar to those seen with angiotensin II, namely, hydrolysis at the Tyr^4–Ile^5 bond and the Arg^2–Val^3 bond (Figure 4).

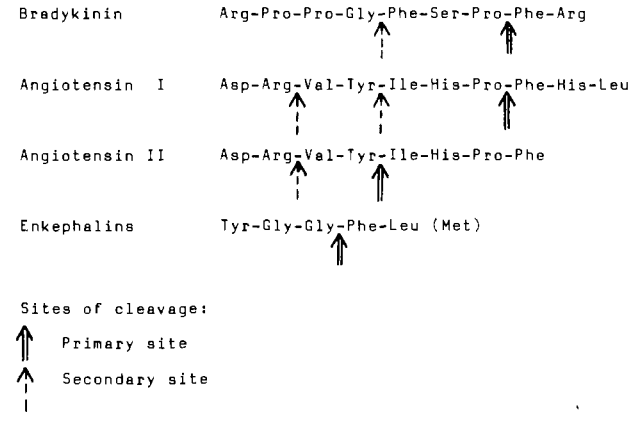

FIGURE 4: Bonds cleaved in peptide substrates by human renal NEP.

Discussion

We report here the first complete purification of human NEP from kidney. The properties of this enzyme are similar to "enkephalinase" activity found in a variety of animal tissues (Hersh, 1982). It is of interest to note that four forms of rat brain "enkephalinase" have been described (Rush & Hersh, 1982). These forms were derived by separation of "enkephalinase" activity into an A_1 and A_2 form by ion-exchange chromatography and further separation of each of these forms by wheat germ lectin chromatography into subforms designated $A_{1,1}$, $A_{1,2}$, $A_{2,1}$, and $A_{2,2}$ (Rush & Hersh, 1982). In the case of the human kidney, the A_1 and A_2 forms of the enzyme could be observed. Pilot studies indicated differences in the carbohydrate content of the rat brain and human kidney enzymes (results not shown), although the renal enzyme is also a glycoprotein containing 10% neutral sugar.

In addition to hydrolyzing Leu^5- and Met^5-enkephalin, human kidney NEP cleaved a number of synthetic substrates in which both the N-terminal and C-terminal amino acids were blocked. A series of thermolysin inhibitors (Nishino & Powers, 1979) also inhibited NEP. Studies with bradykinin and angiotensin I and II showed the human enzyme cleaves bonds adjacent to the N terminal of phenylalanine, leucine, isoleucine, and valine. These results further support the proposal that NEP is a neutral metalloendopeptidase (Hersh, 1982; Fulcher et al., 1982) rather than a dipeptidyl carboxypeptidase as originally suggested (Schwartz et al., 1981).

The high levels of NEP (~0.1% of the total protein) found in the human kidney indicate that both NEP and CE are major constituents of this organ. Although both enzymes cleave the same bond in substrates such as the enkephalins and bradykinin, this observation is perhaps fortuitous since the two enzymes exhibit a rather different specificity toward other substrates. CE cleaves a dipeptide from the C terminus of substrates, while NEP cleaves at the amino side of hydrophobic amino acids. Although both enzymes are metalloenzymes and as such are inhibited by chelating agents and dithio compounds, including dimercaptopropanol, they can be readily differentiated on the basis of inhibition by dipeptide derivatives such as captopril, which is relatively specific for CE (Cushman & Ondetti, 1980), and phosphoramidon and thiorphan, which are relatively specific for NEP (Hudgin et al., 1981). Thus the active sites of the two enzymes differ.

Human kidney NEP did not convert angiotensin I to II but initially cleaved angiotensin I at Pro^7–Phe^8. Angiotensin II was not hydrolyzed by CE (Yang et al., 1970). However, NEP inactivated it by the hydrolysis of the Tyr^4–Ile^5 bond. This cleavage yielded N- and C-terminal tetrapeptide fragments. After a prolonged incubation period, the 1–4 tetrapeptide concentration decreased, giving rise to two new peaks as analyzed by HPLC. No further hydrolysis of the 5–8 tetrapeptide peak was detected. Since no free tyrosine was ob-

served, the products of the secondary cleavage were presumed to be Asp[1]–Arg[2] and Val[3]–Tyr[4]. Although NEP cleaves bonds adjacent to phenylalanine, it does not inactivate angiotensin II by the release of Phe[8]; thus, it does not act as a carboxypeptidase. This was also indicated by the finding that it cleaves Bz-Gly-Phe-Arg but does not release a significant amount of leucine from Bz-Gly-His-Leu.

Other potential substrates of human NEP are substance P and neurotensin (Almenoff et al., 1981; Mumford et al., 1981). NEP and CE both act as "enkephalinase" and "kininase" by cleaving a C-terminal dipeptide from the biologically active substrates. However, they differ in their affinity for these substrates. The K_m of enkephalins with CE is about 0.14–1 mM (Benuck & Marks, 1980; Stewart et al., 1981), depending on the source of enzyme, while Leu[5]-enkephalin has a K_m of $\sim$70 μM with human NEP. The K_m of human or rabbit NEP (Benuck & Marks, 1980) with bradykinin is 120 μM while that of CE was estimated to be 1.0 μM (Erdös, 1979). Thus, at high substrate concentrations, NEP hydrolyzes bradykinin faster than CE, while at low, physiological levels of bradykinin, cleavage by CE would be favored. CE can also be inhibited by high concentrations of kinins. Such inhibition of CE by substrates and dipeptide products of substrates has been demonstrated before (Igic et al., 1972; Yang & Erdös, 1967). In bioassay, when bradykinin was used in the micromolar range, the rate of inactivation by human CE and NEP was similar. If one assumes that both enzymes are located close to each other on the brush border of the proximal tubules and possibly elsewhere, captopril used in vivo could be only partially effective in prolonging the half-life of bradykinin. Captopril inhibits only kininase II, while NEP acting as a kininase would not be affected by this inhibitor.

Benuck & Marks (1980) also noted Phe-Arg liberation by a partially purified rabbit "enkephalinase". Almenoff et al. (1981) reported the hydrolysis of bradykinin by a purified "enkephalinase" from bovine pituitary. Employing a 21-h incubation period, this group observed cleavage of bradykinin at both the Gly[4]–Phe[5] and Pro[7]–Phe[8] bonds. We found that cleavage at the Pro[7]–Phe[8] bond is the important step in the inactivation of bradykinin. Hydrolysis of the Gly[4]–Phe[5] bond occurs in the biologically inactive heptapeptide products, and does not appear to be of significance in the inactivation of the intact bradykinin molecule.

A similar situation also appears to occur in the hydrolysis of angiotensin I and II. Although there are four potential cleavage sites in angiotensin I and II (Arg[2]–Val[3], Val[3]–Tyr[4], Tyr[4]–Ile[5], and Pro[7]–Phe[8]), only one is observed in the intact molecule: Pro[7]–Phe[8] in angiotensin I and Tyr[4]–Ile[5] in angiotensin II.

The activity of CE is restricted by the size of the peptide substrate; it is less active with longer peptides. For example, CE on the surface of endothelial cells cleaves the nonapeptide bradykinin faster than the decapeptide kallidin or the undecapeptide Met-Lys-bradykinin (Johnson & Erdös, 1977). The hog kidney CE cleaves the hexa-, hepta-, and octapeptide derivatives of angiotensin I faster than the intact decapeptide (Chiu et al., 1976). Comparing the kinetic constants for the hydrolysis of bradykinin and Bz-Gly-Phe-Arg indicates the active site of NEP can accommodate longer substrates than CE, which cleaved the shorter peptide substrates faster. It was suggested by Rush et al. (R. Rush, M. Mitas, T. Tanaka, J. Powers, and L Hersh, unpublished results) that NEP has an extended active site. This is also corroborated by the finding that hog CE cleaved the C-terminal dipeptide of the B chain of insulin very slowly (Igic et al., 1972), while this peptide was the substrate most readily hydrolyzed by NEP (Fulcher et al., 1982). Both enzymes are present in high concentration in the kidney and intestine (Erdös, 1979; Danielsen et al., 1980; Fulcher et al., 1982) and probably concentrated on the brush border of the proximal tubules where they can complement each other's action on substrates ranging from oligopeptides to much longer peptide chains. Since both enzymes are also on the membranes of neuroepithelial cells of the brain (Fulcher et al., 1982; Schwartz et al., 1981; Defendini et al., 1982), such a tandem activity should be considered in the CNS as well.

Registry No. CE, 9015-82-1; NEP, 52660-20-5; enkephalinase, 70025-49-9; Leu[5]-enkephalin, 58822-25-6; Suc-Ala-Ala-Phe-MNA, 85613-77-0; Abz-Ala-Tyr-Leu-Ala-Gly-Nba, 85613-78-1; bradykinin, 58-82-2; Bz-Gly-Phe-Arg, 73167-83-6.

References

Almenoff, J., Wilk, S., & Orlowski, M. (1981) *Biochem. Biophys. Res. Commun. 102*, 206–214.

Almquist, R. G., Chao, W. R., Ellis, M. E., & Johnson, H. L. (1980) *J. Med. Chem. 23*, 1392–1398.

Arregui, A., Lee, C. M., Emson, P. C., & Iversen, L. L. (1979) *Eur. J. Pharmacol. 59*, 141–144.

Benuck, M., & Marks, N. (1980) *Biochem. Biophys. Res. Commun. 95*, 822–828.

Blumberg, S., Vogel, Z., & Alstein, M. (1981) *Life Sci. 28*, 301–306.

Chiu, A. T., Ryan, J. W., Stewart, J. M., & Dorer, F. E. (1976) *Biochem. J. 155*, 189–192.

Craves, F. B., Law, P. Y., Hunt, C. A., & Loh, H. H. (1978) *J. Pharmacol. Exp. Ther. 206*, 492–506.

Cushman, D. W., & Ondetti, M. A. (1980) *Prog. Med. Chem. 17*, 41–105.

Danielsen, M. E., Vyas, J. P., & Kenny, A. J. (1980) *Biochem. J. 191*, 645–648.

Defendini, R., Zimmerman, E. A., Weare, J. A., Alhenc-Gelas, F., & Erdös, E. G. (1982) *Adv. Biochem. Psychopharmacol. 33*, 271–280.

Dubois, M., Gillie, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.

Dupont, A., Cusan, L., Garon, M., Alvarado-Urbina, G., & Labrie, F. (1977) *Life Sci. 21*, 907–914.

Erdös, E. G. (1979) *Handb. Exp. Pharmacol., Suppl. 25*, 427–487.

Erdös, E. G., & Sloane, E. M. (1962) *Biochem. Pharmacol. 11*, 585–592.

Erdös, E. G., Johnson, A. R., & Boyden, N. T. (1978) *Biochem. Pharmacol. 27*, 843–845.

Fulcher, I. S., Matsas, R., Turner, A. J., & Kenny, A. J. (1982) *Biochem. J. 203*, 519–522.

Gorenstein, C., & Snyder, S. H. (1979) *Life Sci. 25*, 2065–2070.

Gorenstein, C., & Snyder, S. H. (1980) *Proc. R. Soc. London, Ser. B 210*, 123–132.

Gross, D. M., Sweet, C. S., Ulm, E. H., Backlund, E. P., Morris, A. A., Weitz, D., Bohn, D. L., Wenger, H. C., Vassil, T. C., & Stone, C. A. (1981) *J. Pharmacol. Exp. Ther. 216*, 552–557.

Grynbaum, A., Kastin, A. J., Coy, D. H., & Marks, N. (1977) *Brain Res. Bull. 2*, 479–484.

Hall, E. R., Kato, J., Erdös, E. G., Robinson, C. J. G., & Oshima, G. (1976) *Life Sci. 18*, 1299–1303.

Hambrook, J. M., Morgan, B. A., Rance, M. J., & Smith, C. F. C. (1976) *Nature (London) 262*, 782–783.

Hersh, L. B. (1982) *Mol. Cell. Biochem. 47*, 35–43.

Hudgin, R. L., Charleson, S. E., Zimmerman, M., Mumford, R., & Wood, P. L. (1981) *Life Sci. 29*, 2593–2601.

Igic, R., Erdös, E. G., Yeh, H. S. J., Sorrells, K., & Nakajima, T. (1972) *Circ. Res. 31*, 51-61.
Johnson, A. R., & Erdös, E. G. (1977) *J. Clin. Invest. 59*, 684-695.
Kapitany, R. A., & Zebrowski, E. J. (1973) *Anal. Biochem. 56*, 361-369.
Kerr, M. A., & Kenny, A. J. (1974a) *Biochem. J. 137*, 477-488.
Kerr, M. A., & Kenny, A. J. (1974b) *Biochem. J. 137*, 489-495.
Laemmli, U. K. (1970) *Nature (London) 22*, 680-685.
Lane, A. C., Rance, M. J., & Walter, D. S. (1977) *Nature (London) 269*, 75-76.
Llorens, C., & Schwartz, J. C. (1981) *Eur. J. Pharmacol. 69*, 113-116.
Llorens, C., Gacel, G., Swerts, J. P., Perdrisot, R., Fournie-Zaluski, M. C., Schwartz, J. C., & Roques, B. P. (1980) *Biochem. Biophys. Res. Commun. 96*, 1710-1717.
Malfroy, B., Swerts, J. P., Guyon, A., Roques, B. P., & Schwartz, J. C. (1978) *Nature (London) 276*, 523-526.
Marks, N., Grynbaum, A., & Neidle, A. (1977) *Biochem. Biophys. Res. Commun. 74*, 1552-1559.
Mumford, R. A., Pierzchala, P. A., Strauss, A. W., & Zimmerman, M. (1981) *Proc Natl. Acad. Sci. U.S.A. 78*, 6623-6627.
Nishino, N., & Powers, J. C. (1979) *Biochemistry 18*, 4340-4347.
Orlowski, M., & Wilk, S. (1981) *Biochemistry 20*, 4942-4950.
Pert, C. B., Pert, A., Chang, J. K., & Fong, B. T. W. (1976) *Science (Washington, D.C.) 194*, 330-332.
Rush, R. S., & Hersh, L. B. (1982) *Life Sci. 31*, 445-451.
Schwartz, J. C., Malfroy, B., & DeLaBaume, S. (1981) *Life Sci. 29*, 1715-1740.
Stewart, T. A., Weare, J. A., & Erdös, E. G. (1981) *Peptides (N.Y.) 2*, 145-152.
Sullivan, S., Akil, H., & Barchas, J. D. (1978) *Commun. Psychopharmacol. 2*, 525-531.
Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science (Washington, D.C.) 178*, 871-872.
Wang, C.-S., & Smith, R. L. (1975) *Anal. Biochem. 63*, 414-417.
Weare, J. A., Gafford, J. T., Lu, H. S., & Erdös, E. G. (1982) *Anal. Biochem. 123*, 310-319.
Yang, H. Y. T., & Erdös, E. G. (1967) *Nature (London) 215*, 1402-1403.
Yang, H. Y. T., & Neff, N. H. (1972) *J. Neurochem. 19*, 2443-2450.
Yang, H. Y. T., Erdös, E. G., & Levin, Y. (1970) *Biochim. Biophys. Acta 214*, 374-376.

Stereoselectivity of Chloroperoxidase-Dependent Halogenation†

K. Ramakrishnan, Mark E. Oppenhuizen, Scott Saunders, and Jed Fisher*

ABSTRACT: The stereoselectivity of chloroperoxidase halogenation of four substrates has been examined. Chloroperoxidase catalyzes the bromination, but not chlorination, of racemic 2-*exo*-methylbicyclo[2.2.1]hept-5-ene-2-*endo*-carboxylic acid (to the δ-lactone) and racemic bicyclo-[3.2.0]hept-2-en-6-one (to the 2-*exo*-bromo-3-*endo*-hydroxybromohydrin). These products are obtained in near quantitative yield and are racemic. The circumstances of the bromination strongly suggest that halogenation does not occur at the active site but rather by chloroperoxidase-catalyzed formation of Br_2 and its release into solution. The inability of chloroperoxidase to halogenate these two alkenes at its active site most probably derives from a steric exclusion from the active site. The stereoselectivity of two additional substrates that undergo active site chlorination was determined. Methionine is quantitatively converted to a 50:50 ratio of the two methionine sulfoxide diastereomers. 2-Methyl-4-propylcyclopentane-1,3-dione is quantitatively chlorinated to 2-chloro-2-methyl-4-propylcyclopentane-1,3-dione. On the basis of optical rotation and proton nuclear magnetic resonance, this product is present as a 40:60 ratio of the racemic diastereomers. It is concluded that active site chlorination by chloroperoxidase proceeds without appreciable stereoselectivity.

Enzyme-catalyzed transfer of an electrophilic halogen to an acceptor molecule is a common biological occurrence. It is an event in the elaboration of secondary metabolites by marine plants; and in eukaryotes, it occurs during the synthesis of the thyroid hormones and during the oxidative destruction of microorganisms by the phagocytes. In these instances, the electrophilic halogen is obtained by a hydrogen peroxide dependent oxidation of the halide anion, catalyzed by a haloperoxidase enzyme. The best studied of these enzymes is the chloroperoxidase of the fungus *Caldariomyces fumago* (Hollenberg & Hager, 1978). Within this organism, chloroperoxidase catalyzes the sequential halogenation, at C-2, of 1,3-cyclopentanedione, on the pathway to the antibiotic caldariomycin (2,2-dichloro-*trans*-1,3-cyclopentanediol; Shaw & Hager, 1959; Beckwith et al., 1963). Hager and co-workers have provided a wealth of information on chloroperoxidase, including aspects of its protein chemistry (Morris & Hager, 1966a,b) coenzyme environment (Rutter & Hager, 1982), specificity (Thomas et al., 1970), kinetic mechanism (Libby et al., 1982), and chemical mechanism of halogen activation and transfer (Hollenberg et al., 1974; Araiso et al., 1981). Chloroperoxidase contains an active site ferriprotoporphyrin IX prosthetic group in an environment similar to that found in the cytochrome P-450 monooxygenases (Dawson et al.,

† From the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received January 1, 1983.* We thank the Petroleum Research Fund (administered by the American Chemical Society) for initial support of this research and the National Institute of General Medical Sciences for sustaining support. Grants from the National Science Foundation in support of the high-field nuclear magnetic resonance and amino acid analyzer facilities of the Department of Chemistry, University of Minnesota, are also acknowledged.

0006-2960/83/0422-3271$01.50/0