Characterization of Proline Endopeptidase from Rat Brain†

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ABSTRACT: A homogeneous proline endopeptidase from rat brain is characterized with respect to its substrate specificity and the residues essential for catalysis. The two fluorogenic substrate analogues tested, pyroglutamylhistidylprolyl-β-naphthylamide and pyroglutamyl(*N*-benzylimidazolyl)-histidylprolyl-β-naphthylamide, have higher V_{max} values (19.5 and 26.9 μmol·min^{-1}·mg^{-1}, respectively) and considerably lower K_m values (0.034 and 0.020 mM, respectively) than pyroglutamylhistidylprolylamide (V_{max} = 2.9 μmol·min^{-1}·mg^{-1} and K_m = 4.1 mM). Both fluorogenic substrates give rise to pH optima and pH–rate profiles similar to those of the amide. Values of K_m and k_{cat} are determined as a function of pH. K_m is pH independent, with the titration curve for $k_{cat}K_m^{-1}$ implicating an active-site residue(s) with a pK_a of 6.2. Proline endopeptidase can be completely inactivated by low concentrations of diisopropyl fluorophosphate with an observed second-order rate constant of 2.5 × 10^4 min^{-1}·M^{-1}. The stoichiometry of the alkylphosphorylation is 0.83 mol/mol of enzyme. The pH dependence of the inactivation by diisopropyl fluorophosphate implicates a residue(s) involved in covalent bond formation having a pK_a of 6.0. These data suggest that proline endopeptidase is a serine proteinase.

Recently, an enzyme capable of deamidating thyrotropin-releasing hormone (TRH)[1] has been purified to apparent homogeneity from rat brain by this laboratory (Rupnow et al., 1979). The deamidation of TRH in brain and hypothalamic extracts appears to be primarily due to an enzyme which has been referred to as proline endopeptidase (Taylor & Dixon, 1980). This enzyme has been demonstrated to be a peptidase capable of cleaving a variety of proline-containing polypeptides by hydrolysis of the peptide bond on the carboxyl side of the proline residue (Taylor & Dixon, 1980). A preliminary report describing a similar enzyme from bovine brain has appeared (Tate, 1978) and an enzyme which cleaves polypeptides on the carboxyl side of proline has been isolated from rabbit brain (Orlowski et al., 1979). These enzymes have a number of characteristics similar to those reported for the rat brain enzyme (Rupnow et al., 1979) and most likely represent the equivalent enzyme from different species. All of these enzymes appear to be of intracellular origin and have similar apparent molecular weights as determined by gel filtration chromatography. Thus, a detailed characterization of the rat brain enzyme would be of mechanistic importance and would also aid in further defining the properties of these proline endopeptidases.

The purification of the rat brain enzyme by this laboratory was significantly aided by the development of a fluorescent assay for the enzyme which utilized compound I (Figure 1). Because substrate analogues may not mimic their respective substrates in all parameters, it was important to establish their validity as substrate analogues of TRH within the range of assay conditions used. Figure 1 shows the structures of the two substrate analogues (i.e., compounds I and II) used in this study as well as that of TRH itself.

In general, the mechanisms of action of intracellular proteinases, endopeptidases in particular, have not been examined to as great an extent as those of their extracellular counterparts. However, it appears that intracellular proteinases may be divided into four major mechanistic classes: serine proteinases containing a "superreactive" serine residue, sulfhydryl proteinases, metalloproteinases whose mechanism requires the involvement of divalent metal cations, and acid proteinases which are active only at low pH values (Barrett, 1977). Assignment of proline endopeptidase to one of the above proteinase classes will indicate the general mechanism and will suggest further detailed studies on its mechanism.

Experimental Procedures

Materials

Pyroglutamylhistidyl[2,3-^{3}H]prolylamide (sp act., 113 Ci·mmol^{-1}) was purchased from New England Nuclear. [1,3-^{3}H]Diisopropyl fluorophosphate (sp act., 6.5 Ci·mmol^{-1}) was purchased from Amersham. Benzyloxycarbonyl-L-alanyl-L-prolyl-4-methoxy-β-naphthylamide was obtained from Enzyme Systems Products. Tosyl-L-phenylalanine chloromethyl ketone, tosyl-L-lysine chloromethyl ketone, *N*-ethylmaleimide, bacitracin, *p*-(hydroxymercuri)benzoate, gramicidin, actinomycin D, Grade V ovalbumin, and EDTA were from Sigma Chemical Co. The diisopropyl fluorophosphate, 1,10-phenanthroline, and CDTA were from Aldrich Chemical Co., while 1,5-phenanthroline was from K and K Laboratories, Inc. Leupeptin and pepstatin A were purchased from Peninsula Laboratories, Inc. Trasylol was obtained from Mobay Chemical Corp. Compounds I and II and TRH were from Bachem, Inc., and were purified as previously described (Rupnow et al., 1979). Soybean trypsin inhibitor and lima bean trypsin inhibitor were from Worthington Biochemical Corp. Nembutal was from Abbott Laboratories. AG 1-X4 anion-exchange resin was obtained from Bio-Rad Laboratories. All other reagents were of the highest purity available.

Methods

Activity Assay and Purification of Proline Endopeptidase. The assay for proline endopeptidase using the fluorogenic TRH analogues pyroglutamyl(*N*-benzylimidazolyl)histidylprolyl-β-

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[1] Abbreviations used: TRH, thyrotropin-releasing hormone; CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CBZ-L-Ala-L-Pro-MNA, benzyloxycarbonyl-L-alanyl-L-prolyl-4-methoxy-β-naphthylamide; DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

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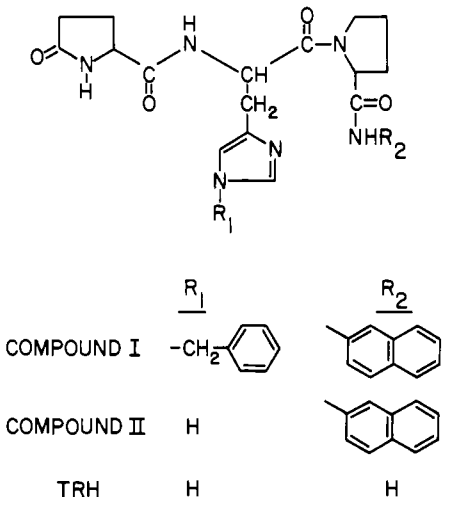

FIGURE 1: Structure of TRH and its fluorogenic analogues. Compound I, pyroglutamyl(*N*-benzylimidazolyl)histidylprolyl-β-naphthylamide; compound II, pyroglutamylhistidylprolyl-β-naphthylamide; TRH, pyroglutamylhistidylprolylamide.

naphthylamide and pyroglutamylhistidylprolyl-β-naphthylamide was performed by using 100 μM substrate in 0.25 M phosphate buffer, pH 7.5, containing 1.0 mM EDTA and 1.0 mM β-mercaptoethanol as described previously (Rupnow et al., 1979). One unit of activity is defined as 1 μmol of substrate hydrolyzed in 1 min. The proline endopeptidase was purified as previously described (Rupnow et al., 1979). The concentrations of both TRH and the two fluorogenic analogues were determined by amino acid analysis following acid hydrolysis in 6.0 N HCl at 105 °C for 24 h. The concentration of substrate was determined relative to a standard alanine solution added prior to hydrolysis. The activity assay using benzyloxycarbonyl-L-alanyl-L-prolyl-4-methoxy-β-naphthylamide was performed with 50 μM substrate as described elsewhere (Taylor et al., 1980).

Reagents Affecting the Activity of Proline Endopeptidase. Solutions for the inactivation of proline endopeptidase were as follows: phenylmethanesulfonyl fluoride and tosyl-L-phenylalanine chloromethyl ketone were in 10% dioxane; 1,5-phenanthroline and 1,10-phenanthroline were in 100% ethylene glycol; diisopropyl fluorophosphate was in 2-propanol. All other reagents were in water. The inactivator (200 μL) and 23 μL of the enzyme (0.08 unit·mL^{-1}) were incubated together with 225 μL of the assay buffer at 37 °C for 10 min. The assay was initiated by adding the enzyme–inhibitor mixture to 0.10 mM substrate in 1.55 mL of 0.25 M phosphate buffer, pH 7.5, containing 1.0 mM EDTA and 1.0 M β-mercaptoethanol.

Results

Comparison of TRH and Its Fluorogenic Analogues as Substrates for Proline Endopeptidase. Figure 2 shows the pH rate profile for compounds I and II and TRH. All three compounds exhibit maximal activity near pH 7.5. Profiles for all three compounds were obtained in both phosphate (Figure 2) and Tris-HCl (data not shown because of their similarity to those noted with phosphate). Although some data points were collected outside the normal buffering range used for the Tris and phosphate solutions, no change in pH occurred during the course of the reaction. It is of interest that substitution of a benzyl group on the nitrogen of the histidine did not appreciably alter the pH dependence of the reaction. This is of particular note because the benzylated substrate (compound I) has a pK_a of 5.26, whereas compound II has a pK_a of 6.36 (data not shown) and one would anticipate that the benzylated

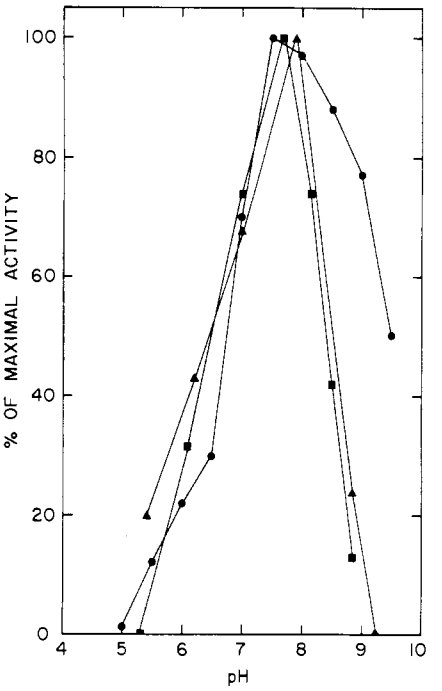

FIGURE 2: The pH–rate profile for the degradation of TRH and its fluorogenic analogues by homogeneous proline endopeptidase. The substrates are compound I (■), II (▲), and TRH (●). The pH–rate profile of the proline endopeptidase using TRH as a substrate was determined by incubation of 10 μL of proline endopeptidase (9.60 × 10^{-3} units·mL^{-1}) with 10 μL of 30 mM TRH, 10 μL of carrier-free pyroglutamylhistidyl[2,3-^{3}H]prolylamide (10 μCi·mL^{-1}), and 70 μL of 0.375 M phosphate titrated to the desired pH. The incubation was carried out at 37 °C for 20 min. The reaction was stopped by heating to 100 °C for 3 min. To this solution were added 100 μL of 0.1 N HCl and 3.0 mL of 10 mM phosphate, pH 5.2. The sample was then applied to a column (8.0 × 0.5 cm) of AG 1-X4. The column was washed with 10 mM phosphate, pH 5.2, and the first 20 mL collected. One milliliter of the eluant was counted for radioactivity. A control, excluding enzyme, was run at each pH value. The pH for each assay was determined by running a parallel assay which excluded radiolabeled TRH and measuring the pH before and after the incubation period. The pH–rate profiles for the fluorogenic analogues of TRH (100 μM) were determined by using 0.25 M Tris-HCl over the pH range 5.3–9.5 and 0.25 M sodium phosphate over the pH range 4.45–7.8. Assays were in triplicate, and the pH was determined after addition of the enzyme to the assay mixture. All assay buffers contained 1 mM β-mercaptoethanol and 1 mM EDTA.

substrate might display an altered pH–rate profile.

While the pH–rate profiles are similar for compounds I and II and TRH, the kinetic parameters for these substrates (Table I) indicate that the fluorogenic analogues are far better substrates than TRH. The K_m values for compounds I and II and TRH are 2.0 × 10^{-5} M, 3.4 × 10^{-5} M, and 410 × 10^{-5} M while the V_{max} values noted for the respective compounds are 26.9, 19.5, and 2.9 μmol·min^{-1}·mg^{-1}. Substitution of the benzyl group on the histidine does not greatly alter the kinetic constants of compound I as compared with its nonbenzylated analogue. However, substitution of the hydrophobic naphthylamide residue for the corresponding amide present in TRH significantly alters the relative affinity and catalytic activity with the enzyme.

Distribution of Proline Endopeptidase. The fluorogenic substrates exhibit pH–rate profiles similar to that of TRH and also appear to be better substrates for the proline endopeptidase than TRH itself. Thyrotropin-releasing hormone has also been shown to be a competitive inhibitor of compound I (Rupnow et al., 1979) and compound II.[2] In addition, electrophoresis

[2] J. E. Dixon and P. C. Andrews, unpublished observation.

Table I: Comparative Kinetic Parameters of Compounds I and II and TRH[a]

substrate	K_m (mM)	sp act. (μmol·min^{-1}·mg^{-1})
compound I	0.020[b]	26.9
compound II	0.034	19.5
TRH	4.1	2.9

[a] The K_m and V_{max} values for TRH were determined by using the method described under Figure 2 and Methods. The TRH concentration was varied from 0.9 to 30.0 mM at pH 7.5. Initial velocities were used and kinetic constants obtained from a Lineweaver–Burk plot of the data by using a least-squares fit to a straight line. The K_m and V_{max} values for compounds I and II were obtained as described by Rupnow et al. (1979). Assays were in triplicate. [b] The discrepancy between this value and the value reported by Rupnow et al. (1979) (i.e., 34 μM as compared to 20 μM reported in this study) is due in part to the different methods employed for measuring the substrate concentration. The previous value (Rupnow et al., 1979) was based upon the weight of the substrate. In this study substrate concentrations were determined by amino acid analysis using an internal standard (see Methods).

Table II: Distribution of Proline Endopeptidase the Rate Nervous System

section no.	brain section	sp act. [units·mg^{-1} (10^{-3})]	total act. (%)	TRH content (pg·mg^{-1})
1	olfactory bulbs	6.8	1.8	6[d]
2	hippocampus	6.5	4.7	
3	pineal[a]	3.2	0.05	
4	thalamus	4.5	1.8	43[c]
5	hypothalamus	3.8	1.4	255[c]
6	caudal colliculus	5.7	2.3	
7	rostral colliculus	5.5	2.0	
8	forebrain plus diencephalon (less brain sections 1–7)	5.1	52.0	
9	medulla oblongata	5.1	7.9	
10	pons	5.9	2.3	15[b]
11	cerebellum	5.9	15.2	1.2[c]
12	spinal cord	2.1	8.4	

[a] Pineal glands were pooled from 50 rats of mixed sex and age, 2 months old. [b] Jackson & Reichlin, 1974. [c] Brownstein et al., 1975. [d] Oliver et al., 1974.

of crude soluble extracts of rat brain showed only one enzyme activity when the gel slices were incubated with compound II (Rupnow et al., 1979). These observations suggested that the hydrolysis of compounds I and II could be used as a reliable index of TRH deamidation and could be used to measure the distribution of the enzyme in crude extracts.

The distribution of the proline endopeptidase in various areas of the rat brain was examined by using compound II as substrate (Table II). The highest specific activity was observed in the olfactory bulbs. However, the lowest specific activity observed was only threefold lower. The ubiquitous distribution of the enzyme is in sharp contrast to the distribution of TRH. A monospecific antibody prepared against the homogeneous enzyme (Taylor et al., 1980) was shown to quantitatively precipitate all enzyme activity capable of hydrolyzing compound II in supernatants prepared from crude brain homogenates, in order to demonstrate that the proline endopeptidase represents the only activity capable of hydrolyzing compound II under the assay conditions used.

Dependence of $k_{cat}K_m^{-1}$ on pH. Values for k_{cat} and K_m were determined at various pH values by using compounds I and II, to gain insight into the mechanism of catalysis of proline endopeptidase. A plot of $k_{cat}K_m^{-1}$ against pH may result in

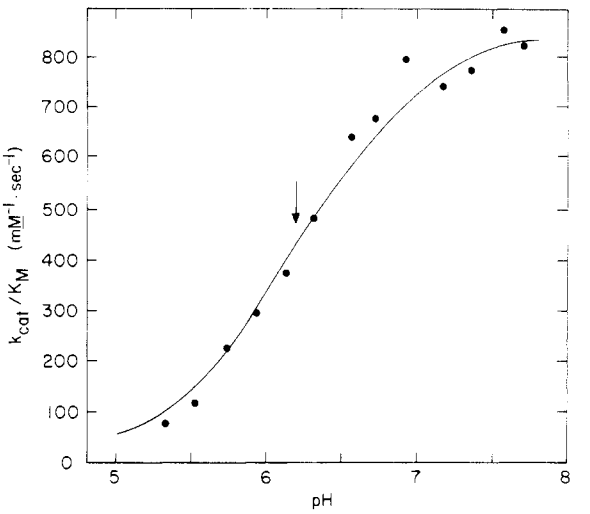

FIGURE 3: Dependence of $k_{cat}K_m^{-1}$ (mM^{-1}·s^{-1}) on pH for proline endopeptidase using compound II as the substrate. The assay was performed as described for the pH–rate profiles, with the exception that the buffer used for the assay was 0.25 M phosphate. Five substrate concentrations were used for each K_m and k_{cat} determination, and all assays were done in triplicate. The line represents a theoretical curve for the titration of a residue having a pK_a of 6.2.

a titration curve for the active-site residues of a given enzyme (Peller & Alberty, 1959). The plot of data for the proline endopeptidase using compound II as substrate has an inflection point at pH 6.2 (Figure 3). The K_m remains constant over the pH range 4.7–7.7, while V_{max} increases from pH 5.35 to pH 7.5 (data not shown). Results identical with those shown in Figure 3 were also obtained when compound I was used as a substrate in plots of $k_{cat}K_m^{-1}$ vs. pH (data not shown). Thus, the ionization of the histidine residue of the substrate has no effect upon either the pH–rate profile or the $k_{cat}K_m^{-1}$ vs. pH profile. The observation that the K_m does not change in the pH range studied indicates that the rate constant for formation of product is much smaller than the rate constant for the dissociation of the enzyme–substrate complex (Cornish-Bowden, 1976). In other words, $K_m \simeq K_S$. Thus, the K_m value represents the dissociation constant for the enzyme–substrate complex when compounds I and II are used as substrates. This observation does not necessarily hold for poorer substrates such as TRH.

Reagents Affecting the Activity of the Proline Endopeptidase. For identification of the particular type or class of peptidase to which the proline endopeptidase belongs, the effect of various reagents on the purified enzyme was examined (Table III). Both sulfhydryl-directed reagents and diisopropyl fluorophosphate (DFP) are effective inactivators of the enzyme. DFP is a specific reagent which is known to inactivate serine proteinases (Means & Feeney, 1971), although it has been shown to react with tyrosine residues in some cases (Means & Wu, 1979; Murachi & Yasui, 1965; Marachi et al., 1970). Reports on sulfhydryl proteinase inactivation by DFP have attributed it to residual diisopropyl chlorophosphate (Gould & Liener, 1965; Murachi & Yasui, 1965). Inactivation of sulfhydryl proteinases by the latter compound does not occur, however, in the presence of low concentrations of thiols (Gould & Liener, 1965). Mercaptoethanol (0.5 mM) was present during the course of the inactivation described in this study. Phenylmethanesulfonyl fluoride is not as effective an inhibitor toward a number of serine proteinases as is DFP (Fahrney & Gold, 1963). The lower percentage of inactivation observed with phenylmethanesulfonyl fluoride is consistent with this observation.

Table III: Reagents Affecting Proline Endopeptidase

reagent	concn (mM)	% inactivation	reagent	concn (mM)	% inactivation
diisopropyl fluorophosphate	0.0067	93	EDTA	0.90	0
diisopropyl fluorophosphate	0.267	99.9	EGTA	0.90	0
iodoacetate	0.90	30	CDTA	0.90	0
iodoacetamide	0.90	53	1,10-phenanthroline	1.10	14
N-ethylmaleimide	0.90	100	1,5-phenanthroline	1.10	21
p-(hydroxymercuri)benzoate	0.90	100	pepstatin	0.67	88
p-(hydroxymercuri)benzoate	0.09	90	leupeptin	0.93	5
tosyl-L-lysine chloromethyl ketone	0.011	25	trasylol	4500[a]	17
tosyl-L-phenylalanine chloromethyl ketone	0.011	33	soybean trypsin inhibitor	0.09[b]	0
phenylmethanesulfonyl fluoride	0.45	34	lima bean trypsin inhibitor	0.09[b]	0
benzamidine	0.90	14			

[a] In kallikrein inactivating units per milliliter. [b] In milligrams per milliliter.

The sulfhydryl-directed reagents, *p*-(hydroxymercuri)-benzoate and *N*-ethylmaleimide, completely inactivate the proline endopeptidase. Two other sulfhydryl-directed reagents, iodoacetamide and iodoacetic acid, are less effective inhibitors. The various chelating agents examined have no effect on enzyme activity, suggesting that the proline endopeptidase does not require divalent metal ions for activity. There is no inhibition of the homogeneous enzyme with either soybean or lima bean trypsin inhibitors. Table III also shows several other reagents which have no effect on enzyme activity or which only have effects at high concentrations (e.g., pepstatin).

It is interesting that 11 μM tosyl-L-lysine chloromethyl ketone (TLCK) and tosyl-L-phenylalanine chloromethyl ketone (TPCK) appear to inactivate the enzyme 25% and 33%, respectively. These two reagents are usually considered to be specific for trypsin-like and chymotrypsin-like proteinases, respectively (Shaw, 1970); however, both reagents will inactivate less specific proteinases such as papain (Whitaker & Perez-Villasenor, 1968). It is surprising that both reagents inactivate an enzyme with a specificity toward proline residues at such low concentrations. Incubation of either reagent with the enzyme results in a loss of enzyme activity which cannot be restored by dialysis or by passing the inactivated enzyme through a gel permeation column. The inactivation by either TPCK or TLCK is time dependent and exhibits apparent saturation effects, and the rate of inactivation is decreased by the addition of TRH to the incubation mixture (data not shown). However, the inactivation by either chloromethyl ketone does not follow simple first-order kinetics and does not result in complete loss of activity. Even at concentrations of 0.3 mM TPCK or TLCK, the enzyme is inactivated only 77% and 78%, respectively, after 30 min. Longer incubations do not result in further inactivation. When the proline endopeptidase was maximally inactivated by either TPCK or TLCK (and the excess reagent removed by dialysis) the modified enzyme showed altered values of K_m and V_{max} when compound II was used as substrate. With TPCK the K_m and V_{max} values are 0.070 mM and 4.9 units·mg^{-1}, respectively, while with TLCK they are 0.067 mM and 7.0 units·mg^{-1}. These observations suggest that these reagents are not affecting the proline endopeptidase in a manner similar to the way they affect trypsin or chymotrypsin.

Inactivation by Diisopropyl Fluorophosphate. An analysis of the rates of inactivation of proline endopeptidase by DFP was undertaken. As illustrated in Figure 4, the inactivation by DFP is rapid at very low concentrations. Pseudo-first-order kinetics are observed to at least 99.3% inactivation. The second-order rate constant was determined to be 2.5 × 10^4 M^{-1}·min^{-1} (insert, Figure 4). This rate constant is larger than those reported for trypsin (3.0 × 10^2 M^{-1}·min^{-1}) and α-chymotrypsin (2.7 × 10^3 M^{-1}·min^{-1}) by Fahrney & Gold (1963). In addition, protection against inactivation by a substrate (TRH) is observed at a concentration fourfold greater than the K_m.

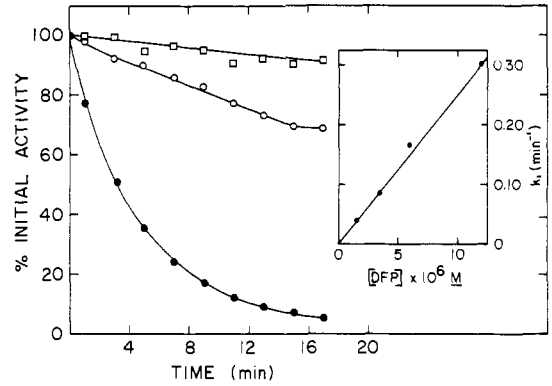

FIGURE 4: Inactivation of proline endopeptidase by DFP. Concentration of DFP was 10 μM (●), 10 μM DFP plus 18 mM TRH (○), and no additions (□). Proline endopeptidase (100 μL of 0.375 unit·mL^{-1}) was incubated at 30 °C with 860 μL of 0.25 M phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol. The DFP was added in 40 μL of 2-propanol. At various time intervals, 100-μL aliquots were removed and assayed with compound II as a substrate. The inset shows the pseudo-first-order rate constants plotted against the DFP concentration.

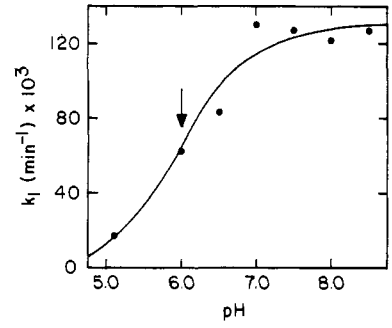

FIGURE 5: Pseudo-first-order rate constant for the inactivation of proline endopeptidase by diisopropyl fluorophosphate as a function of pH. The pH profile was carried out in 0.215 M phosphate buffer containing 0.022 unit·mL^{-1} enzyme at 30 °C. DFP was added in 2-propanol (final concentration of 2-propanol was 4% v/v). The loss of activity in the absence of DFP was determined at each pH. Aliquots removed at various time intervals were assayed for activity with CBZ-L-Ala-L-Pro-4-MNA as substrate. Dilution of the aliquot in the presence of the substrate prevented further inactivation by DFP during the course of the activity assay. The solid line represents the theoretical curve for the titration of a residue having a pK_a of 6.0.

The pH–rate dependence of the first-order rate constant for inactivation by DFP passes through an inflection point at pH

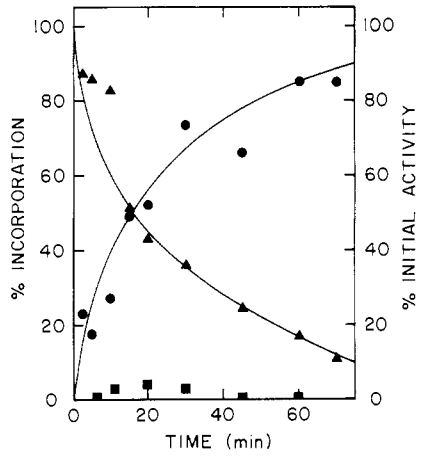

FIGURE 6: Time course for labeling of proline endopeptidase by [1,3-^{3}H]diisopropyl fluorophosphate. The time course of isotope incorporation is indicated (●). The time course of activity loss is also shown (▲). The time course and stoichiometry of ^{3}H incorporation were determined as follows: 135 μL of 0.29 unit·mL^{-1} proline endopeptidase was added to 315 μL of 0.020 M phosphate, pH 7.5, and 2 μL of 770 μM [^{3}H]DFP (6.5 Ci·mmol^{-1}) at 0 °C. At various time intervals, simultaneous aliquots were removed for determination of activity and degree of incorporation. Dilution of the aliquot in the presence of saturating substrate concentration prevented further inactivation during the course of the assay. Aliquots for the determination of the degree of labeling were quenched by addition of 25 μL of 40 mM unlabeled DFP. The non-protein-bound radioactivity was removed from the quenched aliquots on a G-25 column equilibrated with 0.02 M phosphate buffer, pH 7.5, containing 1 mg·mL^{-1} ovalbumin. Column fractions were collected in scintillation vials and counted in a Beckman LS 8100 scintillation counter by using Beckman Ready-Solv EP. The labeling attempt in the presence of TRH was carried out at 18 mM TRH. The heat-inactivated enzyme had been immersed for 10 min in a boiling water bath prior to reacting with [1,3-^{3}H]DFP as described above (■).

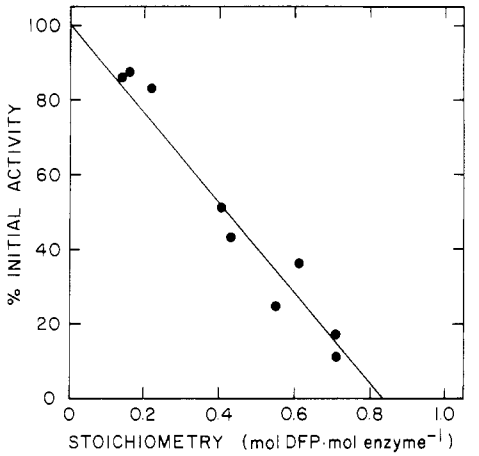

FIGURE 7: Stoichiometry of incorporation of label into proline endopeptidase. The data were obtained as described for Figure 6. The stoichiometry is based on the specific activity reported by Rupnow et al. (1979) and has been corrected for the substrate CBZ-L-Ala-L-Pro-4-MNA.

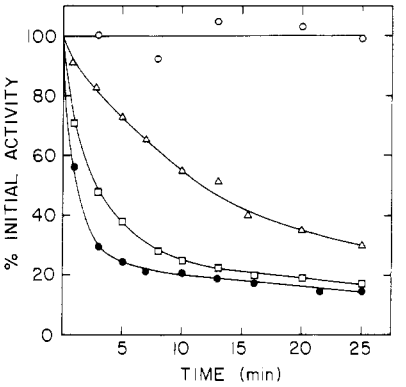

FIGURE 8: Inactivation of proline endopeptidase by iodoacetamide. Concentrations of iodoacetamide were 1.5 mM (△), 6.0 mM (□), 10 mM (●), and 3.0 mM plus 2.1 mM TRH (○). Proline endopeptidase (100 μL of 0.046 unit·mL^{-1}) was incubated at 30 °C with 100 μL of iodoacetamide and 800 μL of 0.25 M phosphate buffer, pH 7.5, containing 1.0 mM EDTA. At indicated time intervals, 100-μL aliquots were removed and assayed with compound II as substrate.

6.0 (Figure 5). The first-order rate constant reaches a maximal value above pH 7.5 of 1.3 × 10^5 min^{-1} at 3 μM DFP.

Radiolabeling of Proline Endopeptidase by [1,3-^{3}H]Diisopropyl Fluorophosphate. The time course for isotope incorporation with subsequent inactivation of the proline endopeptidase using [1,3-^{3}H]diisopropyl fluorophosphate is shown in Figure 6. The incorporation of isotope is concomitant with the loss of activity. The observed rate of incorporation of the label is decreased in the presence of 18 mM TRH, suggesting substrate protection of the enzyme against incorporation in the presence of the tripeptide. No incorporation of label is observed for the heat-inactivated enzyme (Figure 6).

Stoichiometry of Incorporation of [1,3-^{3}H]Diisopropyl Fluorophosphate into Proline Endopeptidase. For determination of the stoichiometry of isotope incorporation into the proline endopeptidase, the level of isotope incorporation (expressed as moles of [1,3-^{3}H]DFP per mole of enzyme) was determined as a function of activity loss (Figure 7). The observed stoichiometry is 0.83 mol of label incorporated per mol of enzyme. The same stoichiometry is observed in the presence or absence of TRH. The stoichiometry is based on the specific activity reported by Rupnow et al. (1979) and has been corrected for the use of the substrate benzyloxycarbonyl-L-alanyl-L-prolyl-4-methoxy-β-naphthylamide (CBZ-L-Ala-L-Pro-4-MNA).

Analysis of the Inactivation by Iodoacetamide. The time course for inactivation by iodoacetamide shown in Figure 8 reveals that complete inactivation of the proline endopeptidase does not occur. Approximately 20% residual activity is observed after 25 min in the presence of 10 mM iodoacetamide. Pseudo-first-order kinetics of inactivation are not observed, suggesting that a single, uniformly reactive residue is not being modified. However, the enzyme is completely protected against inactivation by 2.1 mM TRH, suggesting that modification of the enzyme by iodoacetamide can be influenced by substrate. Proline endopeptidase which has been completely modified by 10 mM iodoacetamide as judged by the time course of inactivation demonstrates both an increased K_m (0.20 mM) and a decreased V_{max} (17 μmol·min^{-1}·mg^{-1}) when compound II was used as substrate. The increase in K_m indicates that all enzyme molecules are modified, suggesting that the residual activity observed does not appear to be due to a small fraction of unmodified enzyme. In addition, it could be demonstrated that the enzyme inactivated to 80% by using the conditions described for Figure 8 would incorporate 0.67 mol of [1,3-^{3}H]DFP per mol of enzyme.

Discussion

The observation that the fluorogenic analogues of TRH have lower K_m values than TRH itself most likely reflects the presence of a hydrophobic binding site for residues on the carboxyl side of the proline. The similar K_m values for the benzylated and the nonbenzylated analogues indicate that the enzyme can accommodate some structural alterations in the position immediately preceding proline. This observation is

consistent with the fact that the rat brain proline endopeptidase will hydrolyze a variety of substrates having neutral, acidic, or basic amino acid residues adjacent to the proline undergoing catalysis (Taylor et al., 1980). The kinetic constants, pH–rate profiles, enzyme activity noted on polyacrylamide gels (Rupnow et al., 1979), and data obtained from immunoprecipitation (Taylor et al., 1980) all suggest that compounds I and II act in a fashion similar to TRH. However, there is reason to believe compounds I and II do not always exactly mimic TRH with respect to hydrolysis by proline endopeptidase. Specifically, the reported K_i for TRH (with respect to compound I) (Rupnow et al., 1979) is considerably lower than the K_m for TRH (4.1 mM). This observation would not be expected for a purely competitive inhibition where a single inhibitor molecule would bind at the active site of the enzyme unless the rate-limiting step for hydrolysis of TRH is different from that for compounds I and II. Under the conditions which were employed for the kinetic evaluation of proline endopeptidase in this study, no behavior incompatible with Michaelis–Menten kinetics was observed.

The pH profile of $k_{cat}K_m^{-1}$ demonstrates that a residue having a pK_a near 6.2 is required in the unprotonated form for activity. While this observation may not be interpreted to mean that the residue titrated is essential for catalysis, the pK_a value affords some information as to the class of proteinase to which the proline endopeptidase belongs. The observed pK_a of 6.2 is higher than that observed for sulfhydryl proteinases [papain at 4.7 (Bender & Brubacker, 1966) and ficin at 4.5 (Whitaker, 1969)] and is similar to that observed for serine proteinases [α-chymotrypsin at 6.6 and trypsin at 6.7 (Mounter et al., 1956)].

The question regarding the general mechanism of proline endopeptidase may be more directly approached through the use of specific modification reagents. The inactivation of proline endopeptidase by the relatively specific reagent DFP allows the residues critical for catalysis to be more closely examined. We have found that treatment of proline endopeptidase with DFP results in the rapid and complete loss of activity. Pseudo-first-order kinetics were observed during the course of inactivation. These data are consistent with modification of a single residue important for activity. The protection against inactivation by DFP conferred by the substrate, TRH, suggests that the residue modified is located at or near the active site. The location of the residue at the active site is supported by the observation that the concentration of TRH required for protection (18 mM) is in the range of the K_m (4.1 mM).

The observed stoichiometry of modification by [1,3-^{3}H]-diisopropyl fluorophosphate of 0.83 residues per mol of enzyme modified supports the suggestion that a single residue is involved in the inactivation. The incorporation of label with concomitant loss of enzyme activity is again consistent with a single residue being modified on the enzyme which in turn results in a loss of enzyme activity.

The pH dependence of the first-order rate constant for the inactivation by DFP suggests that a residue having a pK_a of 6.0 must be in the unprotonated form in order for the alkylphosphorylation to occur. The pK_a observed is much lower than those observed in cases where tyrosyl residues have been shown to be modified [lysozyme at 10.2 (Murachi et al., 1970), stem bromelain at 8.5 (Murachi & Yasui, 1965), and human serum albumin at 8.3 (Means & Wu, 1979)] and is near the pK_a values reported for serine proteinases [α-chymotrypsin at 6.6, trypsin at 6.7 (Mounter et al., 1956), and carboxypeptidase Y at 6.0 (Hayashi et al., 1975)]. This observation suggests that the residue which reacts with DFP is a seryl residue having an arrangement of residues in the active site similar to other serine proteinases such as trypsin and chymotrypsin. The similarity between the pK_a of the residue essential for the catalysis and the pK_a of the residue involved in reactivity with DFP suggests that the same residue is involved in both cases and that the inactivation by DFP is enzymatically promoted. The data are consistent with the mechanism observed for serine proteinases in which the protonation of the histidyl residue at the active site results in a large decrease in the nucleophilicity of the neighboring seryl residue (Kraut, 1977).

While the data obtained for the inactivation by DFP are clearly consistent with a serine proteinase, the data presented in Table II indicate that sulfhydryl-directed reagents can also affect the activity of proline endopeptidase. The inactivation by iodoacetamide is not complete even at high concentrations of the reagent. The alkylating reagents TPCK and TLCK exhibit similar properties and, in addition, give apparent saturation kinetics. While protection of the enzyme by TRH in the presence of each of these three reagents may indicate that the residue(s) alkylated is located near the active site, alternative explanations cannot be ruled out. In addition, it is possible that the residue(s) alkylated by iodoacetamide, TLCK, and TPCK is not cysteine since it has been observed that both iodoacetamide (Vallee & Riordan, 1969) and chloromethyl ketones (Powers, 1978) will modify residues other than cysteine. On the other hand, *p*-(hydroxymercuri)benzoate appears to be a specific reagent toward cysteine residues at the concentrations and pH values employed here (Means & Feeny, 1971). Complete loss of activity upon treatment with *p*-(hydroxymercuri)benzoate does not, however, mean that a cysteine residue is directly involved in the mechanism. A noncatalytic cysteine residue which appears to be at or near the active site of certain serine proteinases and whose modification results in loss of activity has been reported (Bai & Hayashi, 1979).

Both the postproline cleaving enzyme from lamb kidney (Yoshimoto et al., 1977) and the proline-specific dipeptidyl aminopeptidase from the same source (Kenny et al., 1976) have been reported to be inactivated by DFP. A partially purified enzyme from bovine pituitary which also deamidates TRH has been reported to be inactivated by DFP (Knisatchek et al., 1980).

The data presented here are consistent with the rat brain proline endopeptidase being a serine proteinase, having a "superreactive" serine at the active site generated via hydrogen bonding to a nearby histidyl residue. The effects of iodoacetamide, TPCK, and TLCK are less clear, however, and should be examined more thoroughly.

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An Essential Methionine in Pig Kidney General Acyl-CoA Dehydrogenase†

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ABSTRACT: The flavoprotein pig kidney general acyl-CoA dehydrogenase contains a single catalytically essential methionine residue/FAD which reacts with iodoacetate at pH 6.6. S-Carboxymethylation of this residue generates an inactive enzyme derivative which retains FAD and the tetrameric structure of the native protein. The derivative binds octanoyl-CoA and palmitoyl-CoA with concomitant perturbation of the flavin chromophore, but the characteristic spectrum of the reduced enzyme·enoyl-CoA complex is not observed. In addition, octanoyl-CoA strongly protects the native enzyme against alkylation with iodoacetate. These results suggest that the methionine residue is within the active center of acyl-CoA dehydrogenase. Carboxymethylation of this residue may disrupt the precise orientation of the substrate required to achieve transfer of reducing equivalents to the flavin. Pig kidney general acyl-CoA dehydrogenase does not contain exposed catalytically essential cysteine residues.

Mammalian acyl-CoA dehydrogenases comprise an important group of closely related flavoproteins which catalyze the insertion of a trans-2,3 double bond in their acyl thioester substrates during β-oxidation. Three classes of dehydrogenases have been isolated from pig liver with overlapping substrate specificities for short (Green et al., 1954), medium (Crane et al., 1956; Hall & Kamin, 1975), and long (Hauge et al., 1956; Hall et al., 1976) chain fatty acyl-CoA thioesters. These dehydrogenases transfer the reducing equivalents generated in this oxidation to a second flavoprotein, electron-transferring flavoprotein (ETF;[1] Crane & Beinert, 1956; Hall et al., 1979), which interacts with the respiratory chain probably at the level of a membrane-bound iron–sulfur flavoprotein (Ruzicka & Beinert, 1977).

We have previously reported the purification and properties of an acyl-CoA dehydrogenase of general specificity from pig kidney (Thorpe et al., 1979). The present study describes the carboxymethylation of an essential methionine in this flavoprotein and some of the properties of the derivatized enzyme.

Experimental Procedures

Materials. Pig kidney general acyl-CoA dehydrogenase was purified as described previously (Thorpe et al., 1979; Thorpe, 1980). All enzyme concentrations refer to bound FAD with the experimentally determined ϵ_{446} value of 15.4 mM^{-1} cm^{-1} (Thorpe et al., 1979). Visible and UV spectra were recorded on a Cary 219 instrument. All buffers contained 0.3 mM EDTA. Where appropriate, iodo[1-^{14}C]acetic acid (Amersham) was diluted to a specific activity of 0.55 μCi/μmol with unlabeled iodoacetic acid from Sigma. Solutions of iodoacetic acid were kept dark. Acyl-CoA thioesters were from P-L Biochemicals, and all other reagents were of analytical grade from commercial sources.

Enzyme Assays. Acyl-CoA dehydrogenase was assayed with octanoyl-CoA by using phenazine methosulfate and dichlorophenolindophenol as described previously (Thorpe et al.,

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[1] Abbreviations used: ETF, electron-transferring flavoprotein; SCM, *S*-carboxymethyl; CoA, coenzyme A.

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