Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Martinez-Carrion, M., & Raftery, M. A. (1973) *Biochem. Biophys. Res. Commun. 55*, 1156.
Martinez-Carrion, M., Šator, V., & Raftery, M. A. (1975a) *Biochem. Biophys. Res. Commun. 65*, 129.
Martinez-Carrion, M., Thomas, J. K., Raftery, M. A., & Šator, V. (1975b) *J. Supramol. Struct. 4*, 373.
Moore, W. M., & Brady, R. N. (1977) *Biochim. Biophys. Acta 498*, 331.
Nickel, E., & Potter, L. T. (1973) *Brain Res. 57*, 508.
Nieva-Gomez, D., & Gennis, R. B. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1811.
Ong, D. E., & Brady, R. N. (1974) *Biochemistry 13*, 2822.
Osborn, M., & Weber, K. (1969) *J. Biol. Chem. 244*, 4406.
Raftery, M. A., Bode, J., Vandlen, R., Michaelson, D., Deutsch, J., Moody, M., Ross, J., & Stroud, R. M. (1975) *Protein–Ligand Interact. Proc. Symp., 1974*, 328.
Raftery, M. A., Vandlen, R. L., Reed, K. L., & Lee, T. (1976) *Cold Spring Harbor Symp. Quant. Biol. 40*, 193.
Šator, V., Raftery, M. A., & Martinez-Carrion, M. (1978) *Arch. Biochem. Biophys. 190*, 57.
Šator, V., Thomas, J. K., Raftery, M. A., & Martinez-Carrion, M. (1979) *Arch. Biochem. Biophys. 192*, 250.
Schimerlik, M., & Raftery, M. A. (1976) *Biochem. Biophys. Res. Commun. 73*, 607.
Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349.
Weil, C. L., MacNamee, M. G., & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997.
Witzemann, V., & Raftery, M. A. (1977) *Biochemistry 16*, 5862.
Witzemann, V., & Raftery, M. A. (1978) *Biochemistry 17*, 3598.
Yamaoka, T., Kashiwagi, H., & Nagakura, S. (1972) *Bull. Chem. Soc. Jpn. 45*, 361.

Purification and Characterization of a Thyrotropin-Releasing Hormone Deamidase from Rat Brain†

John H. Rupnow,‡ William L. Taylor,§ and Jack E. Dixon*,¶

ABSTRACT: This report describes the purification of a rat brain thyrotropin-releasing hormone (TRH) deamidating enzyme to apparent homogeneity. Criteria for purity include sodium dodecyl sulfate and disc gel electrophoresis, as well as isoelectric focusing (p*I* = 4.5). Enzyme purification was facilitated by development of a rapid and sensitive continuous assay using the substrate L-pyroglutamyl-N^{im}-benzylhistidyl-L-prolyl-β-naphthylamide, which, upon hydrolysis of the naphthylamide, results in the appearance of the fluorescent product, β-naphthylamine (βNA). With this substrate the homogeneous enzyme had a specific activity of 14.5 μmol of βNA min^{-1} mg^{-1}. The only peptide product formed was shown to be L-pyroglutamyl-N^{im}-benzylhistidyl-L-proline. Hydrolysis of [L-*prolyl*-2,3-^{3}H]TRH was shown to yield L-pyroglutamyl-L-histidyl-L-proline as the only radiolabeled product. Characterization of the brain deamidase by gel filtration chromatography and sodium dodecyl sulfate gel electrophoresis indicated that the enzyme consists of a single polypeptide chain having molecular weights of 70 000 and 73 500, respectively. Rat brain TRH deamidase has an apparent K_m of 34 μM, and a pH optimum between 7 and 8 using L-pyroglutamyl-N^{im}-benzylhistidyl-L-prolyl-β-naphthylamide as a substrate. With this substrate, TRH was shown to be a competitive inhibitor with an apparent K_i of 120 ± 20 μM.

Thyrotropin-releasing hormone (TRH)[1] is secreted from neurons located in the hypothalamus and travels via the portal vein to the anterior pituitary. There it interacts with receptors on the anterior pituitary to ultimately release thyrotropin, prolactin, and, under certain conditions, growth hormone (Reichlin et al., 1976). In addition to its localization in the hypothalamus, TRH has been demonstrated by bioassay and radioimmunoassay to be present in the extrahypothalamic areas of the brain (Jackson, 1978). The function of the extrahypothalamic TRH activity is unclear, although it has been suggested that it may function as a neurotransmitter (Schaeffer et al., 1977).

Several investigators have reported the presence of TRH inactivating enzymes in the hypothalamus, as well as other areas of the brain (Bauer & Kleinkauf, 1974; Taylor & Dixon, 1976; Prasad & Peterkofsky, 1976, and references therein). The role these TRH peptidases play in regulating the intra- and extracellular concentrations of the hormone is not yet understood. In an effort to begin to understand the role TRH-peptidases play in hormone regulation, this laboratory has purified to apparent homogeneity a TRH-deamidating

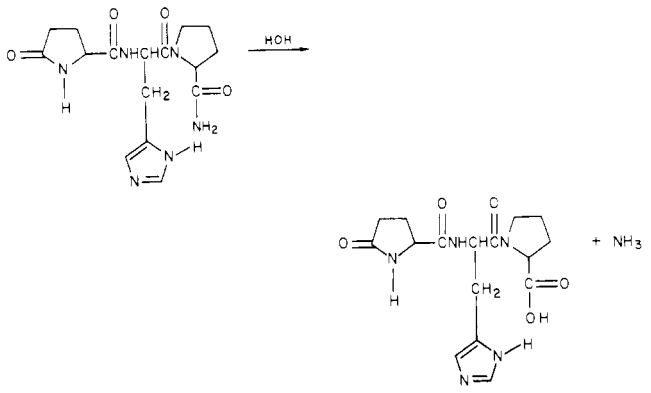
† From the Department of Biochemistry, Purdue University, West Lafayette, Indiana 47906. *Received September 28, 1978.* This work was supported in part by a Research Grant, AM 18849, from the National Institutes of Arthritis, Metabolism, and Digestive Diseases. This is Journal Paper No. 7351 from the Purdue University Agricultural Experiment Station, West Lafayette, IN 47906.

‡ John H. Rupnow is a postdoctoral National Institutes of Health trainee (AM 0594601).
§ William L. Taylor is a predoctoral National Institutes of Health trainee (GM 07076).
¶ Jack E. Dixon is the recipient of a Research Career Development Award, AM 00218, from the National Institutes of Health.

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; pGlu, pyroglutamic acid; TRH, thyrotropin-releasing hormone (pGlu-His-Pro-NH_2); TRH(Bz-His)-βNA, pGlu-(N^{im}-benzyl-L-His)Pro-β-naphthylamide; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; βNA, β-naphthylamine.

0006-2960/79/0418-1206$01.00/0

enzyme from rat brain. The reaction catalyzed by the enzyme is shown below.



The enzyme hydrolyzes the amide bond of the hormone, forming pGlu-His-Pro, a metabolite which has little or no biological activity (Guillemin et al., 1971).

Experimental Procedures

Materials. [L-*prolyl*-2,3-^{3}H]TRH was purchased from New England Nuclear and TRH from Bachem Fine Chemicals Co. Wistar rats were from the Department of Biochemistry's (Purdue University) animal colony. All gel filtration and ion-exchange resins except Bio-Gel A-0.5m (Bio-Rad) were products of Pharmacia Fine Chemicals. Acrylamide for disc gel electrophoresis was purchased from Canalco. All proteins used to calibrate either gel filtration columns or polyacrylamide gels were commercial preparations of the highest quality available and were used without further purification. Ampholine was obtained from LKB, and β-naphthylamine and Fast Green FCF were products of Sigma.

Synthesis of pGlu-(N^{im}-benzyl-L-His)-Pro-β-naphthylamide. L-Pyroglutamyl-N^{im}-benzyl-L-histidyl-L-prolyl-β-naphthylamide [TRH(Bz-His)-βNA] was prepared for this laboratory by Bachem Fine Chemicals Co., Torrance, CA.

The product of the synthesis was analyzed for homogeneity using a Varian high pressure liquid chromatograph (Model 8500) equipped with a Partsil-10 column employing butanol:acetic acid:water (4:1:1) as the solvent. A major peak representing 89% of the product, based on absorbance at 250 nm, was resolved. A sample taken from fractions under this peak was dried under nitrogen, hydrolyzed for 24 h in 6 N HCl at 110 °C and subjected to amino acid analysis on a Durrum amino acid analyzer (Model D-500). Glutamic acid, benzylhistidine, and proline were found to be present in equal molar concentrations.

The heterogeneous substrate was used during purification of the TRH-deamidase. Preparative high pressure liquid chromatography was used to purify the substrate for kinetic studies and TRH-deamidase product analysis.

Protein Determinations. During purification of the rat brain TRH-deamidase, protein concentrations were determined by relating the absorbance at 280 nm to a bovine serum albumin standard curve. The protein concentrations used to determine subcellular TRH-deamidase localization were carried out according to Hartree's (1972) modification of the Lowry et al. (1951) method using bovine serum albumin as the standard.

Assay for Enzyme Activity. Enzyme activity was measured by monitoring β-naphthylamine formation from the TRH analogue TRH(Bz-His)-βNA. The appearance of this fluorescent product was monitored continuously with a recorder-equipped American Instruments fluorescence spectrometer (Model 125) using excitation and emission wavelengths of 335 and 410 nm, respectively. Relative fluorescence was converted to nanomoles of β-naphthylamine using a standard curve. A stock TRH(Bz-His)-βNA substrate solution was prepared by dissolving 9.7 mg in 5 mL of 1 N acetic acid. The pH was adjusted to 4.5 with NaOH and the volume brought to 10 mL with assay buffer (0.25 M sodium phosphate, pH 7.5, 1 mM EDTA, and 1 mM 2-mercaptoethanol).

Routinely, 100 μL of stock substrate solution was incubated at 37 °C with aliquots of enzyme and sufficient assay buffer (usually 1.8 mL) to afford a final volume of 2 mL.

Polyacrylamide Gel Electrophoresis. Electrophoretic analysis of native enzyme was performed using 7.5% acrylamide gels which had been preelectrophoresed at pH 8.9. Electrophoretic separations were achieved at 4 °C using Tris–glycine buffer, pH 8.3, and a constant current of 3 mA/tube.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed by the method of Weber et al. (1972). Samples were run at a constant 6 mA/tube in 7.5% gels. All gels were stained for protein with 0.2% Coomassie brilliant blue.

Enzyme activity in polyacrylamide gels following electrophoresis under nondissociating conditions was detected by slicing the gel into 3-mm sections. Each slice was placed in a tube containing 50 μL of TRH(Bz-His)-βNA stock solution and 1.95 mL of assay buffer. The tubes were incubated overnight at room temperature and examined for fluorescence as previously described.

Subcellular Localization of TRH-deamidase Activity. A 200-g male rat was sacrificed by decapitation and the brain excised. The brain was homogenized in 15 mL of 0.01 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol for 15 s using a Polytron homogenizer at setting 4. The homogenate was centrifuged at 600*g* for 10 min to yield the crude extract. A small portion of the crude extract was retained for activity and protein assays and the remainder centrifuged at 10000*g* for 10 min. The 10000*g* pellet was retained and the supernatant centrifuged at 27000*g* for 30 min. The 27000*g* supernatant fluid and pellet were retained for quantitation of TRH-deamidase activity and protein concentration. All pellet fractions were suspended in a minimal volume of 0.01 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. All fractions were assayed for TRH-deamidase activity as previously described, and for protein concentration according to the method of Hartree (1972).

Isoelectric Focusing. The purified rat brain TRH-deamidase was subjected to isoelectric focusing at 4 °C in 4.0% acrylamide gels containing 1.9% pH 3–6 range and 0.1% pH 3–10 range Ampholine according to the method of Righetti & Drysdale (1971). Current was maintained at 1 mA/tube until the voltage had risen to 400 V. Voltage was thereafter maintained at 400 V for 6–7 h with total time of electrophoresis limited to 9 h. Gel lengths were recorded and either stained in Fast Green FCF (Riley & Coleman, 1968), or cut into 5-mm sections for determination of pH gradient. The gradient was measured at room temperature with a digital pH meter after gel sections had been macerated in 0.5 mL of distilled water. The gels stained for protein were scanned at 600 nm.

Identification of the Products of TRH(Bz-His)-βNA Degradation by TRH-deamidase. Stock substrate was purified by high pressure liquid chromatography according to the procedure previously described. The substrate (2.0 mg) was then dissolved in 2.25 mL of 0.1 N acetic acid. The pH was adjusted to 7.5 with NH_4OH and the volume brought to 2.5

Table I: Purification of Rat Brain TRH-deamidase

step		vol (mL)	total act. (μmol of βNA/min)	total protein (mg)	sp act. (μmol of βNA min^{-1} mg^{-1})	purifn	recovery (%)
1	homogenate	650	62.1	15700	0.004		
2	centrifuged homogenate	710	48.4	6360	0.008	2.0	78
3	ammonium sulfate fractionation	95.0	39.4	1120	0.035	8.8	63
4	chromatography on phenyl-Sepharose	40.0	29.9	52.5	0.570	142	48
5	chromatography on DEAE-Sepharose	45.0	20.6	9.00	2.29	572	33
6	gel filtration on Sephacryl S-200	14.2	9.41	1.20	7.84	1960	15
7	gel filtration on Bio-Gel A-0.5m	6.0	8.55	0.71	12.0	3010	14
8	second chromatography on phenyl-Sepharose	11.6	3.05	0.21	14.5	3625	5

mL with distilled water. A 50-μL aliquot of purified enzyme (0.17 mg/mL) in 0.01 M sodium phosphate buffer containing 1 mM EDTA and 1 mM 2-mercaptoethanol was added and the reaction mixture was incubated for 3 h at 37 °C. The hydrolysate was lyophilized to dryness, dissolved in 1-butanol:acetic acid:water (4:1:1), and centrifuged at 8000*g* to remove denatured protein. The incubation mixture, authentic TRH(Bz-His)-βNA, and β-naphthylamide were spotted on Silica Gel G (Analabs) and developed in 1-butanol:acetic acid:water (4:1:1) or 1-butanol:acetic acid:water:pyridine (30:6:20:24). Peptides were visualized according to the method of Pataki (1963) except plates were sprayed with a solution containing 1% starch and 1% KI. The β-naphthylamine was identified using an ultraviolet light.

The major peptide product of the hydrolysis was recovered by preparative thin-layer chromatography. The plate was scraped at the location which corresponded to the R_f of the major peptide product. The peptide was eluted with methanol and the silica was removed by centrifugation. The methanol was removed under nitrogen and the residue dissolved in 0.1 N acetic acid and lyophilized to dryness. The amino acid composition of the peptide was determined as previously described.

Identification of the Products of [L-*prolyl*-2,3-^{3}*H*]*TRH Degradation by the Deamidase.* The incubation mixture consisted of 2 μCi of [L-*prolyl*-2,3-^{3}H]TRH (21.8 Ci/mol) in 20 μL of 0.01 M sodium phosphate, pH 7.5, containing 0.15 M NaCl and an 80-μL aliquot of enzyme containing approximately 500 ng of deamidase. Following incubation at 37 °C for 1 h, the reaction mixture was frozen and lyophilized and then dissolved in 200 μL of methanol. This solution was applied to either Silica Gel G or cellulose thin-layer chromatography plates (Eastman). The silica plates were developed in chloroform:methanol:ammonia (125:75:25) or 1-propanol:30% ammonia (7:3). Thin-layer electrophoresis was performed at pH 3.5 for 35 min at 500 V in pyridine:acetic acid:water (1:10:189) or at pH 6.5 for 45 min at 400 V (pyridine:acetic acid:water, 100:3:879). Samples of TRH, pGlu-His-Pro and proline were chromatographed or electrophoresed with the hydrolysate. The histidine-containing compounds were visualized with Pauly reagent and proline was identified with ninhydrin. Radioactivity was determined after scraping 0.5-cm sections of the plates into scintillation vials and adding a toluene-based scintillation fluor.

Results

Subcellular Localization of TRH-deamidase. Prasad & Peterkofsky (1976) presented evidence that the TRH-deamidase in guinea pig brain was a soluble enzyme. The subcellular distribution of the TRH-deamidase from rat brain was carried out as described in Methods. The 27000*g* supernatant fluid contained 93% of the total deamidase activity, and the specific activity of this fraction was six times that of either the 10000*g* or 27000*g* pellet fractions. These results are consistent with the observations of Prasad & Peterkofsky (1976).

Purification of the TRH-deamidase. Steps used in the purification of rat brain TRH-deamidase are described below and summarized in Table I. All steps were carried out at 4 °C.

(*1*) *Extraction.* One hundred rats (Wistar strain), weighing approximately 300 g each, were decapitated and the brains (191 g) excised. The brains were homogenized in 400 mL of 0.1 M sodium phosphate, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol for 30 s on a Polytron homogenizer at setting 4. The resulting homogenate was centrifuged at 25000*g* for 1 h. The supernatant fluid was retained and the pellet washed once with 200 mL of extraction buffer and recentrifuged as above. The two supernatant fluids were pooled.

(*2*) *Ammonium Sulfate Fractionation.* The pooled supernatant fluids were fractionated by stepwise addition of solid ammonium sulfate. Protein precipitating between 0 and 50% ammonium sulfate saturation was collected by centrifugation at 25000*g* for 30 min and discarded. Protein precipitating between 50% and 85% ammonium sulfate saturation was collected as above and dissolved in 70 mL of 0.1 M sodium phosphate, pH 7.5, containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and sufficient ammonium sulfate to achieve 25% saturation.

(*3*) *Chromatography on Phenyl-Sepharose.* The protein from step 2 was applied to a 1.5 × 50 cm phenyl-Sepharose Cl-4B column which had been packed and equilibrated with 25% saturated ammonium sulfate, 0.01 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. Equilibration buffer was passed over the column at a flow rate of 20 mL/h until the optical density of the eluate (A_{280}) approached zero. Bound protein was eluted with a 400-mL linear gradient of increasing ethylene glycol to 50% (v:v) and decreasing ammonium sulfate to zero percent saturation. Fractions catalyzing the hydrolysis of more than 2 nmol of βNA min^{-1} $(10\ \mu L)^{-1}$ (Figure 1) were pooled and dialyzed against three, 2-L changes of 0.025 M sodium phosphate, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. This step resulted in the removal of a large amount of protein from the TRH-deamidase and yielded an

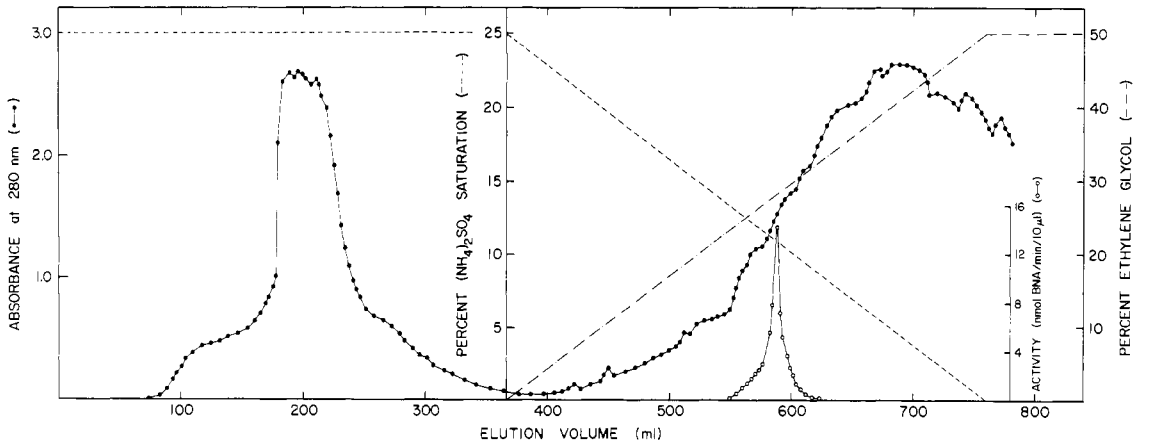


FIGURE 1: Phenyl-Sepharose chromatography. Absorbance at 280 nm and enzyme activity are represented by closed (●) and open (○) circles, respectively. The fractions between 562 and 602 mL of the elution volume were pooled and used in the following step of the purification.

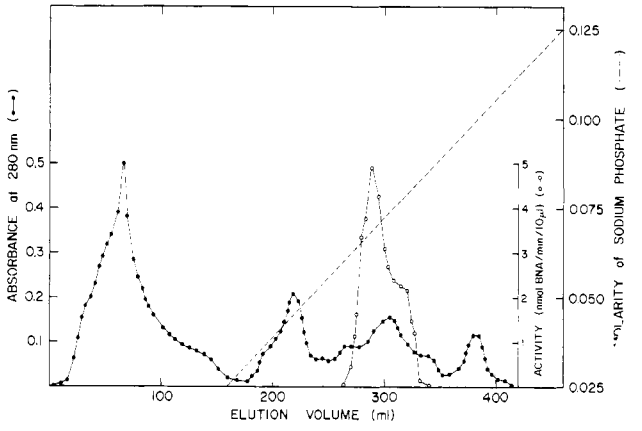


FIGURE 2: DEAE-Sepharose chromatography. Absorbance at 280 nm and enzyme activity are represented by closed (●) and open (○) circles, respectively. The fractions between 280 and 325 mL of the elution volume were pooled and used in the following step of the purification.

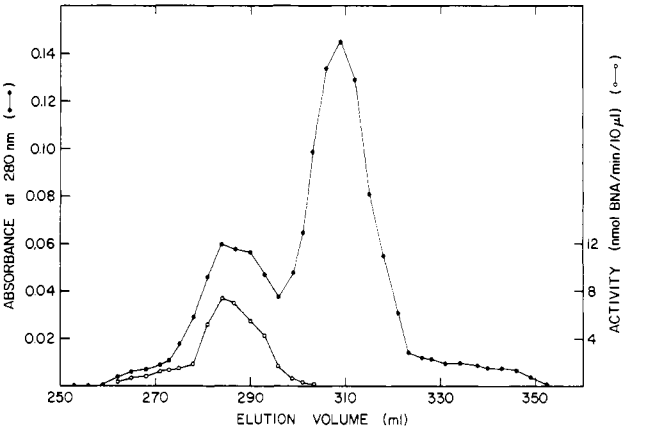


FIGURE 3: Sephacryl S-200 gel filtration. The absorbance at 280 and the enzyme activity are represented by closed (●) and open (○) circles, respectively. The fractions between 279 and 293 mL of the elution volume were pooled and used in the following step of the purification.

overall purification of 142-fold (Table I).

(*4*) *Ion-Exchange Chromatography on DEAE-Sepharose.* The dialyzed protein from step 3 was applied to a 1.5 × 30 cm DEAE-Sepharose column which had been packed and equilibrated with 0.025 M sodium phosphate, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. Equilibration buffer was passed over the column at a flow rate of 30 mL/h until the optical density (A_{280}) of the eluate approached zero. Bound protein was eluted with a 300-mL sodium phosphate linear gradient to 0.1 M. Deamidase activity was eluted between 0.06 and 0.09 M sodium phosphate (Figure 2). Fractions which catalyzed the liberation of more than 2.0 nmol of βNA min^{-1} (10-μL aliquot)$^{-1}$ were pooled; at this stage an overall purification of 572-fold and a recovery of 48% had been achieved.

(*5*) *Gel Filtration on Sephacryl S-200.* A 2.5 × 120 cm column of Sephacryl S-200 was equilibrated with 0.01 M sodium phosphate, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The pooled fractions from step 4 were concentrated to 2 mL (Amicon, PM-10), applied to the column, and eluted at a flow rate of 20 mL/h (Figure 3). Fractions which catalyzed the liberation of more than 2.0 nmol of βNA min^{-1} (10-μL aliquot)$^{-1}$ were pooled and yielded an overall purification of 1960-fold and a recovery of 15%.

(*6*) *Final Purification Steps.* Disc gel electrophoresis showed that the protein resulting from step 5 was not pure. The protein solution was concentrated to 1.5 mL (Amicon, PM-10) and passed over a 1.5 × 130 cm Bio-Gel A-0.5m column using 0.1 M sodium phosphate, pH 7.5, buffer containing 1 mM EDTA and 1 mM 2-mercaptoethanol as the eluent. Fractions which catalyzed the liberation of more than 4 nmol of βNA min^{-1} (5-μL aliquot)$^{-1}$ were pooled. This step was followed by a second phenyl-Sepharose column run according to the procedure previously described and resulted in homogeneous protein when evaluated by several criteria.

The rat brain TRH-deamidase had a specific activity of 14.5 μmol of βNA min^{-1} (mg of protein)$^{-1}$. The results shown in Table I indicated an overall purification of 3625-fold with a recovery of 5%.

The enzyme was stored at 4 °C in 0.01 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 25% glycerol, and 12% ammonium sulfate. The enzyme can be stored under these conditions for 30 days with less than a 10% loss in activity.

Evidence of Homogeneity. The purified protein was examined for homogeneity using acrylamide gel electrophoresis under nondissociating and dissociating conditions. The protein migrated as a single band under dissociating conditions in 7.5% gels containing $NaDodSO_4$ (Figure 4) and under nondissociating conditions in 7.5% gels (Figure 4). Electrophoresis of rat brain homogenate according to the procedure described in Methods resulted in a single band of activity with mobility corresponding to that of stained, purified protein (Figure 4). Additional support for a homogeneous protein was obtained from isoelectric focusing experiments which resulted in a single major protein band.

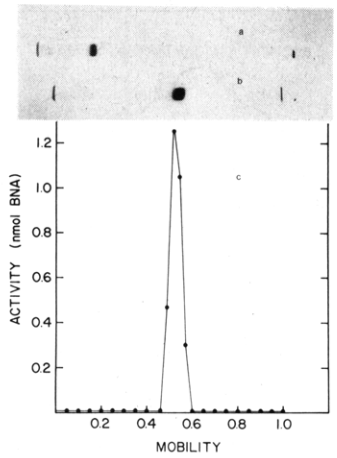


FIGURE 4: Polyacrylamide gels of (a) purified rat brain TRH-deamidase (10 μg) denatured in the presence of $NaDodSO_4$ and 2-mercaptoethanol and (b) native purified TRH-deamidase (15 μg). (c) The enzyme activity profile obtained after electrophoresis of the 25000*g* supernatant rat brain homogenate.

Molecular Weight. Two methods were employed to estimate the molecular weight of the enzyme. The enzyme was examined by its elution volume of Sephacryl S-200 according to the method of Andrews (1965) and was found to have an apparent molecular weight of 70 000. The protein standards used included ribonuclease, chymotrypsinogen, ovalbumin, bovine serum albumin, and aldolase. Subunit composition of the enzyme was determined using gel electrophoresis in the presence of $NaDodSO_4$ according to the procedure of Weber et al. (1972). A plot of the relative mobilities of several protein standards vs. the log of their molecular weights was linear and the relative mobility of the brain TRH-deamidase on 7.5% gels was characteristic of a protein having a molecular weight of 73 500. Collectively the molecular weight determinations also suggest that the native enzyme consists of a single polypeptide chain.

Isoelectric Focusing. Following isoelectric focusing and staining with Fast Green FCF, the enzyme appeared as a major band having an isoelectric point of 4.5 with a shoulder of minor absorbance at pH 4.35 (data not shown).

Determination of pH Optimum. The pH optimum for TRH rat brain deamidase was determined by incubating 100-μL aliquots of enzyme solution with 100 μL of stock TRH(Bz-His)-βNA substrate and 1.8 mL of 0.25 M sodium phosphate, or 0.25 M Tris-HCl buffer, each containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The enzyme is active between pH 4.8 and pH 8.8, and has a pH optimum between 7.0 and 8.0 in both phosphate and Tris buffer. The maximum velocity in phosphate buffer is near pH 7.4, while in Tris buffer it is approximately 7.7 (data not shown). Enzyme incubated at pH 9.0 for 10 min and then adjusted to pH 7.5 and assayed gave no evidence of activity. However, complete recovery of enzyme activity was achieved when enzyme was incubated for 10 min at pH 6.0 and then adjusted to pH 7.5 before assaying.

Kinetics of TRH Brain Deamidase with TRH(Bz-His)-βNA. Concentrations of TRH(Bz-His)-βNA used to determine the Michaelis constant were 7.5 to 100 μM. The 2-mL reaction mixtures containing the desired substrate concentration and TRH-deamidase (0.22 μg) in 0.25 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol were incubated at 37 °C and assayed according to the procedure described in Methods. The K_i for TRH was determined by assaying in the presence of 250, 125, and 62.5 μM TRH. The K_m for TRH-deamidase and the K_i for TRH were calculated by Lineweaver–Burk analysis.

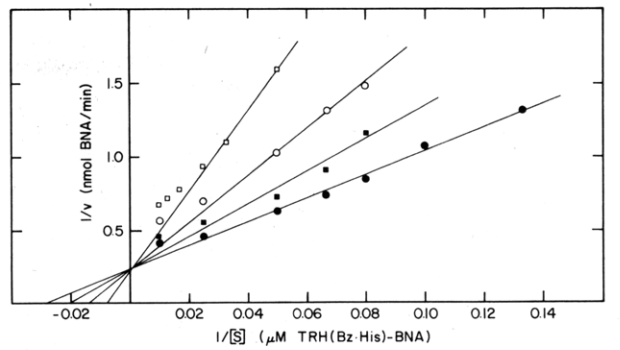


FIGURE 5: Lineweaver–Burk plot of rat brain TRH-deamidase. The closed circles (●) show the enzyme activity measured in the absence of TRH. ■, ○, and □ represent activities in the presence of 62.5, 125, and 250 μM TRH, respectively.

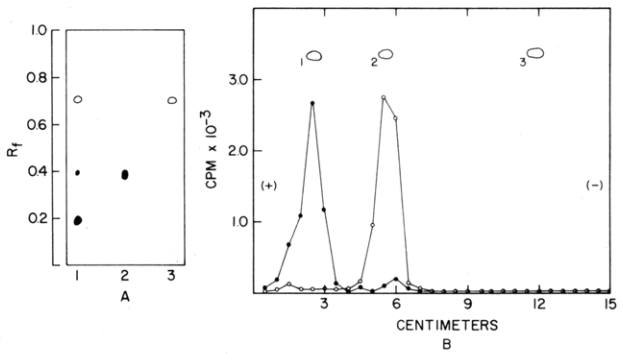


FIGURE 6: (A) Thin-layer chromatography analysis of TRH-deamidase hydrolysis of TRH(Bz-His)-βNA. Chromatograms include hydrolysate (1), unhydrolyzed substrate (2), and β-naphthylamine (3) developed in 1-butanol:acetic acid:water, 4:1:1. (B) Electrophoretic separation of the metabolites of [L-*proyl*-2,3-^{3}H]TRH following its reaction with the rat brain TRH-deamidase. Electrophoretic product analysis at pH 3.5. [L-*prolyl*-2,3-^{3}H]TRH incubated with (●) and without (○) rat brain TRH-deamidase. Standards used were pGlu-His-Pro (1), TRH (2), and proline amide (3).

The TRH-deamidase follows Michaelis–Menten kinetics using TRH(Bz-His)-βNA as the substrate (Figure 5). The Lineweaver–Burk plot was linear between 7.5 and 40 μM TRH(Bz-His)-βNA (Figure 5). Substrate inhibition was apparent at concentrations in excess of 40 μM. The enzyme has a K_m for TRH(Bz-His)-βNA of 34 μM and, assuming that there is 1 mol of active site per 73 500 g of protein, a turnover number of 1.4×10^3 min^{-1} can be calculated. The turnover number results in an apparent higher specific activity than the value reported in Table I since the routine enzyme assay is performed at 0.1 mM substrate, a concentration which results in substrate inhibition. In the presence of TRH, the enzyme exhibits apparent competitive inhibition when data at substrate concentrations less than 30 μM are considered, yielding an apparent K_i for TRH of 120 ± 20 μM.

Product Analysis of the TRH-deamidase. The products of TRH(Bz-His)-βNA hydrolysis were examined by thin-layer chromatography and amino acid analysis. Incubation of the enzyme with the TRH analogue as described in Methods resulted in the formation of a peptide whose R_f differed from that of unhydrolyzed substrate along with a single fluorescent product that comigrated with authentic β-naphthylamine (Figure 6A). The peptide product of the hydrolysis was isolated by preparative thin-layer chromatography and subjected to amino acid analysis. Glutamic acid, benzylhistidine, and proline were present in equimolar amounts. Product analysis was also carried out on the peptides following chromatography in 1-butanol:acetic acid:water:pyridine

(20:6:20:25). Again the above-described products were resolved.

Product analyses using [L-*prolyl*-2,3-^{3}H]TRH resulted in a single radioactive product that comigrated with authentic pGlu-His-Pro using thin-layer electrophoresis at pH 3.5 (Figure 6B). Similarly, a single product identified as pGlu-His-Pro was observed upon electrophoresis at pH 6.5 and also upon thin-layer chromatography on silica gel in 1-propanol:30% ammonium hydroxide (7:3) and in chloroform:methanol:ammonium hydroxide (125:75:25) (data not shown).

Discussion

Use of the substrate TRH(Bz-His)-βNA greatly facilitated purification of the TRH-deamidase. This assay provides a direct, sensitive, and continuous means of following enzyme activity. Previous methods of monitoring TRH degradation were often based upon a decrease in [L-*prolyl*-2,3-^{3}H]TRH concentration (Taylor & Dixon, 1976; Prasad & Peterkofsky, 1976). The homogeneous enzyme purified using the substrate analogue of TRH also hydrolyzes [L-*prolyl*-2,3-^{3}H]TRH as shown in Figure 6B. Although the homogeneous enzyme catalyzes the hydrolysis of both [L-*prolyl*-2,3-^{3}H]TRH and TRH(Bz-His)-βNA, it should be emphasized that the latter compound is a substrate analogue of TRH and, therefore, comparative properties of the two substrates with TRH-deamidase should be made with caution.

The purification of the TRH-deamidase affords an homogeneous protein as judged by migration on $NaDodSO_4$ and polyacrylamide gels and isoelectric focusing. The apparent molecular weights of the protein as determined by $NaDodSO_4$ polyacrylamide gels and gel permeation chromatography were 73 500 and 70 000, respectively. These results indicate that the enzyme is a monomer.

Enzymes which cleave the peptide bond on the carboxyl side of proline have been purified from rat liver and kidney (Hopsu-Havu & Glenner, 1966; Hopsu-Havu & Sarimo, 1967; Hopsu-Havu et al., 1968) as well as from lamb kidney (Koida & Walter, 1976). The enzyme purified from rat liver and kidney is an exopeptidase with an apparent molecular weight as determined by ultracentrifugal analysis of 230 000. This proline dipeptidyl aminopeptidase also appears to be a dimer. In contrast, the lamb kidney enzyme has an apparent molecular weight of 115 000 as determined by gel filtration chromatography and is also most likely a dimer. Both of these enzymes appear to be serine proteases (Yoshimoto et al., 1978). The enzyme described in this report clearly has a number of properties which are quite distinct from the two enzymes described above. In addition, results obtained in this laboratory indicate that the homogeneous brain TRH-deamidase is very sensitive to sulfhydryl-directed reagents such as iodoacetamide and *N*-ethylmaleimide.[2] The postproline cleaving enzyme (Yoshimoto et al., 1978) obtained from lamb kidney is very resistant to iodoacetamide. The lamb kidney endopeptidase hydrolyzes a wide variety of -Pro-X peptides. The dipeptidyl aminopeptidase which was originally thought to be an exopeptidase has recently been shown to also possess endopeptidase activity (Kenny et al., 1976). Although there is limited information available concerning the substrate specificity of the TRH-deamidase, it should be noted that it will cleave the Pro-NH_2 bond of TRH as well as TRH(Bz-His)-βNA, but is inactive toward a wide variety of L-amino acid β-naphthylamides, including L-prolyl-β-naphthylamide.[3]

In addition to the studies described above, a rabbit brain endopeptidase which hydrolyzes the Pro^7-Phe^8 peptide bond of bradykinin has been described (Oliveira et al., 1976). This enzyme, however, was not obtained in homogeneous form and, therefore, its relationship to the TRH-deamidase or other endo- or exopeptides which cleave on the carboxyl side of proline is difficult to determine.

Previous studies indicated that incubation of TRH with brain extracts abolished the biological and immunological activity of the hormone (Bauer & Lipmann, 1976; McKelvy et al., 1976). Taylor & Dixon (1976) demonstrated that an enzyme present in the high-speed supernatant fraction of rat hypothalamic extracts degraded TRH to pGlu-His-Pro and that this activity was very susceptible to sulfhydryl inactivating reagents. Prasad & Peterkofsky (1976) characterized a TRH-deamidase present in hamster hypothalamic extracts and observed that thyroid-stimulating hormone inhibited the deamidase activity. Recently, Tate (1978) reported the purification of an enzyme from bovine brain which deamidates TRH. This enzyme was inhibited by sulfhydryl-directed reagents and appears to inactivate luteinizing hormone releasing hormone. The studies described above may involve similar enzymes obtained from different sources, all of which are capable of degrading TRH.

The putative role that the brain endopeptidase plays in regulating the concentration of TRH or other brain peptides or proteins remains unsolved. The possibility that this enzyme has a functional role in addition to the deamidation of thyrotropin-releasing hormone should not be overlooked, for there are numerous examples of polypeptides which must undergo proteolytic processing in the brain in order to generate biologically active polypeptides (Li & Chung, 1976). The procedures for purification of the rat brain TRH-deamidating enzyme described herein should afford the possibility of answering more specific questions about the physiological role of this enzyme.

References

Andrews, P. (1965) *Biochem. J. 96*, 595.

Bauer, K., & Kleinkauf, H. (1974) *Z. Physiol. Chem. 355*, 1173.

Bauer, K., & Lipmann, F. (1976) *Endocrinology 99*, 230.

Guillemin, R., Burgus, R., & Vale, W. (1971) *Vitam. Horm. (New York) 29*, 1–39.

Hartree, E. F. (1972) *Anal. Biochem. 48*, 422.

Hopsu-Havu, V., & Glenner, G. (1966) *Histochemie 7*, 197.

Hopsu-Havu, V., & Sarimo, S. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 150.

Hopsu-Havu, V. K., Rintola, P., & Glenner, G. (1968) *Acta. Chem. Scand. 22*, 229.

Jackson, I. M. D. (1978) *Res. Publ., Assoc. Res. Nerv. Ment. Dis., 56*, 217.

Kenny, A. J., Booth, A. G., George, S. G., Ingram, J., Kershaw, D., Wood, C. J., & Young, A. R. (1976) *Biochem. J. 157*, 169.

Koida, M., & Walter, R. (1976) *J. Biol. Chem. 251*, 7593.

Li, C. H., & Chung, D. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1145.

Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.

McKelvy, J. F., LeBlanc, P., Laudes, C., Perrie, S., Grimm-Jorgensen, Y., & Kordon, C. (1976) *Biochem. Biophys. Res. Commun. 73*, 507.

[2] J. Rupnow & J. E. Dixon, unpublished results.

[3] J. E. Dixon, unpublished results.

Oliveria, E., Martins, A., & Camargo, C. (1976) *Biochemistry 15*, 1967.
Pataki, G. (1963) *J. Chromatogr. 12*, 541.
Prasad, C., & Peterkofsky, A. (1976) *J. Biol. Chem. 251*, 3229.
Reichlin, S., Saperstein, R., Jackson, I., Boyd, A., & Patel, Y. (1976) *Annu. Rev. Physiol. 38*, 389.
Righetti, P., & Drysdale, J. W. (1971) *Biochim. Biophys. Acta 236*, 17.
Riley, R., & Coleman, M. (1968) *J. Lab. Clin. Med. 72*, 1974.
Schaeffer, J., Brownstein, M., & Axelrod, J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3579.
Tate, S. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1780 (Abstract).
Taylor, W. L., & Dixon, J. E. (1976) *Biochim. Biophys. Acta 444*, 428.
Weber, J., Pringle, J., & Osborn, M. (1972) *Methods Enzymol. 26*, 3.
Yoshimoto, T., Fischl, M., Orlowski, R., & Walter, R. (1978) *J. Biol. Chem. 253*, 3708.

Structure of Lactate Dehydrogenase Inhibitor Generated from Coenzyme†

Jean-François Biellmann,* Claude Lapinte, Erich Haid, and Guenter Weimann

ABSTRACT: Two inhibitors of lactate dehydrogenase generated during NADH storage have been isolated by chromatography. One is a dimer of the dinucleotide where the AMP moiety is unmodified. The other is also generated from NAD^+ in the presence of a high concentration of phosphate ions at alkaline pH. This inhibitor was proved to be the addition compound of one phosphate group to position C-4 of the nicotinamide ring of NAD^+ by NMR spectroscopy, enzymatic cleavage, and dissociation to NAD^+ at neutral pH. This compound is a competitive inhibitor with respect to NAD^+ in the presence of the lactate dehydrogenase with a K_i of 2×10^{-7} M. The interaction of this inhibitor with lactate dehydrogenase is discussed relative to the structure of this enzyme.

Inhibitors of dehydrogenases appearing during storage of $NAD(P)^+$ and NAD(P)H have been known for a long time (Dalziel, 1961, 1962, 1963; Fawcett et al., 1961; Fine et al., 1962; Silverstein, 1965; Holman et al., 1966; Strandjord & Clayson, 1966; McComb & Gay, 1968; Härtel et al., 1968; Klotzsch et al., 1969; Babson & Arndt, 1970; Berry et al., 1973; Gerhardt et al., 1974; Gallati, 1976a,b,c; Loshon et al., 1977; Margolis et al., 1977).[1] The inhibitors are responsible for erratic kinetic results and falsify analytical determinations. Earlier, ADPR had been characterized as the inhibitor for alcohol dehydrogenase present in the NAD^+ preparation (Dalziel, 1961, 1962; Yonetani, 1963). For the dihydrofolate reductase from *Lactobacillus casei*, a dependence on the source and the treatment of NADPH has been noticed for the enzymic activity and was attributed to inhibitors present in NADPH (Williams et al., 1977).

Very strong inhibitors for lactate dehydrogenases from various origins appear during the storage of NADH in the presence of air or moisture as well as by repeatedly freezing and thawing NADH solutions (Fawcett et al., 1961; Silverstein, 1965; Strandjord & Clayson, 1966; McComb & Gay, 1968; Härtel et al., 1968; Klotzsch et al., 1969; Berry et al., 1973; Gerhardt et al., 1974; Gallati, 1976a,c). It is not known whether these different treatments yielded the same compounds. Since ADPR, adenosine diphosphate, and AMP are poor inhibitors for the lactate dehydrogenases (Geyer, 1968; McPherson, 1970), the inhibitors have presumably different structures. Isolation procedures of these compounds have been published but no definite structures have been established for them (Fine et al., 1962; Strandjord & Clayson, 1966; McComb & Gay, 1968).

Since lactate dehydrogenase is of major analytical importance in clinical diagnoses (Schmidt & Schmidt, 1976), it is therefore essential to avoid the formation of these inhibitors.[2] For this reason a structural determination of these inhibitors was undertaken. We shall report on the structure of one inhibitor, which was also formed from NAD^+ and phosphate at alkaline pH (Gallati, 1976a,b,c), and on the partial structure of another one. The third inhibitor has not been studied here.

Materials

NAD^+, NADH, NADPH, AMP, NMN, ADPR, sodium pyruvate, and alkaline phosphatase from calf intestine (grade I), phosphodiesterase from snake venom (Crotalus), lactate dehydrogenase from rabbit muscle, and yeast alcohol dehydrogenase were obtained from Boehringer Mannheim Corp.; Sephadex (R) G-15 and QAE-Sephadex (R) A-25, from Pharmacia, Uppsala, Sweden.

Methods

Spectral Data. *^1H NMR spectra* were determined at 250 MHz with a Cameca spectrometer or at 90 MHz with a

† From the Institut de Chimie, Université Louis Pasteur de Strasbourg, Strasbourg, France (J.-F.B. and C.L.), and from Boehringer Mannheim GmbH, Biochemica Werk Tutzing, Tutzing, West Germany (E.H. and G.W.). *Received May 1, 1978.*

[1] Abbreviations used: AMP, adenosine monophosphate; NMN^+, nicotinamide mononucleotide; ADPR, adenosine diphosphate ribose; NAD^+, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Inh_1, inhibitor eluted first after NADH by chromatography on a QAE-Sephadex column; Inh_2, inhibitor eluted second after NADH by chromatography on a QAE-Sephadex column.

[2] Generally, a ratio of absorbance at 260–340 nm of less than 2.32 is a good indication of an inhibitor-free NADH preparation.

0006-2960/79/0418-1212$01.00/0