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Active-Site Studies on Rabbit Liver Nicotinamide Deamidase†

Leigh D. Albizati* and Jerry L. Hedrick

ABSTRACT: The possibility that control of the Preiss–Handler pathway of NAD biosynthesis is at the nicotinamide deamidase catalyzed reaction has stimulated investigations into the kinetic, physical, and structural properties of this enzyme. This work describes the number of, and relation between, the active centers for amidase and esterase activity of rabbit liver nicotinamide deamidase. The presence of three active sites was determined by reaction of the site-specific reagents diisopropyl fluorophosphate (DFP) and carbobenzoxyamido-2 phenylethyl chloromethyl ketone (ZPCK) with the enzyme and from binding studies. DFP treatment inhibited both esterase and amidase activities, whereas ZPCK inhibited amidase activity but did not affect esterase activity or amide binding. The relation between the catalytic sites was further investigated by kinetic and binding studies using competitive inhibitors (nicotinic acid, *p*-nitrophenol, benzoic acid, hydrocinnamic acid, and 3-indolepropionic acid). The pH dependence of the amidase and esterase activities established that the esterase activity has a higher pH optimum than the amidase activity. Ionizable groups on the ES complex with pK_a values of 5.6–5.8 affect both activities. Chemical characterization of the enzyme included COOH-terminal studies (0.9 mole of leucine/mole of protein as measured by carboxypeptidase action) and sulfhydryl group determination (0.8 mole of SH/mole of enzyme). It was concluded that: (1) the ester and amide substrates share three binding sites on the enzyme, one of which is not equivalent to the other two; (2) both the esterase and amidase activities involve seryl residues; (3) the amidase activity requires histidyl residues which are not involved in ester hydrolysis or amide binding; and (4) a nonhistidine nucleophilic group may be involved with the seryl residues in the esterase activity.

The widespread occurrence of the enzyme nicotinamide deamidase (Preiss and Handler, 1958a,b; Imsande, 1961; Joshi and Handler, 1962; Bernheim, 1967; Pallini *et al.*, 1965; Petrack *et al.*, 1963, 1965; Kirchner *et al.*, 1966; Marki and Greengard, 1966) and the possibility that control of the Preiss–Handler pathway of NAD biosynthesis may occur *in vivo* at this reaction (Greengard *et al.*, 1963, 1965, 1969) have stimulated investigations into the physical, kinetic, structural, and control properties of the enzyme (Su *et al.*, 1969). Of particular interest was the finding that nicotinamide deamidase from rabbit liver also catalyzes the hydrolysis of a variety of ester substrates (Su *et al.*, 1969). This dual activity of the enzyme is analogous to that of a number of other enzymes (*e.g.*, chymotrypsin, trypsin, carboxypeptidase-A, elastase, thrombin) which also hydrolyze amide or peptide bonds and ester bonds. Furthermore, nicotinamide deamidase activity was affected by the site-specific reagents DFP and ZPCK[1] (Su and Chaykin, 1971; Gillam *et al.*, 1972), thus implicating seryl and histidyl residues in the active center of the enzyme. These amino acid residues have been well established as part of the active centers of other amidase and esterase enzymes such as trypsin, chymotrypsin (Schoellmann and Shaw, 1963), elastase (Naughton *et al.*, 1960; Smillie and Hartley, 1964), plasmin (Groskopf *et al.*, 1969), and others (Dixon, 1966) using the same or related site-specific inhibitors.

This work was undertaken to elucidate the nature of the active center(s) of rabbit liver nicotinamide deamidase, determine the number of active sites and substrate binding sites, and ascertain if the active center for amidase activity is identical with the active center for esterase activity.

Materials and Methods

Chemicals. [7-^{14}C]Nicotinamide (specific activity 42 mCi/mmole) was purchased from New England Nuclear. It was

† From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616. *Received June 14, 1971.* Supported in part by U. S. Public Health Service Research Grant No. AM 1178 from the National Institute of Arthritis and Metabolic Diseases.

* This work is taken from a dissertation submitted by Leigh D. Albizati to the Graduate Division of the University of California, Davis, in partial fulfillment of the requirements for the degree of Doctor of Philosophy and was supported by U. S. Public Health Service Training Grant No. GM 119. Present Address: Department of Biological Chemistry, University of California School of Medicine, Davis, California 95616.

[1] The abbreviations used are: ZPCK, carbobenzoxyamido-2-phenylethyl chloromethyl ketone; DIP, diisopropylphosphoryl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl fluorophosphate.

further purified as described previously (Kirchner *et al.*, 1966) and then diluted with unlabeled nicotinamide to a specific activity of $\sim 2.4 \times 10^4$ cpm/μmole. Fresh rabbit livers were obtained from a local slaughterhouse. Hydroxylapatite was obtained from Bio-Rad Laboratories; polyethylene glycol 6000 from J. T. Baker; [^{32}P]DFP from Nuclear-Chicago and Amersham-Searle. ZPCK and [^{14}C]ZPCK (specific activity 3.21×10^7 cpm/mmole) were the generous gifts of Dr. Elliott Shaw, Biology Department, Brookhaven National Laboratory, Upton, N. Y. All other chemicals were the purest grade commercially available.

Preparation of Nicotinamide Deamidase. Two methods were used to prepare microsomal acetone powder from fresh rabbit livers. Method I has been described by Su *et al.* (1969). Method II is based on the precipitation of microsomes in a system adapted from Albertsson (1960; Su and Chaykin, 1971).

Purification of the enzyme was performed essentially according to the method of Su *et al.* (1969). Several changes made in scaling up the procedure did not affect the homogeneity of the isolated enzyme. The specific activity of the freshly prepared enzyme was occasionally 20–30% higher than that reported by Su *et al.* (1969) but after several days frozen storage all preparations stabilized at 3.4–4.5 μmoles/mg hr. (a) Centrifugations were done at 16,000*g* for 30 min at the temperatures indicated previously. (b) The initial extraction involved 200 g of microsomal acetone powder and 4 l. of 0.2 M glycine buffer, pH 8.9, done in four 50-g batches. (c) The pH 5.0 precipitation step was omitted. (d) The deamidase activity was precipitated between 35 and 55% acetone (v/v) in the final step. The precipitate was dialyzed against 1 mM potassium phosphate buffer, pH 6.8. Approximately 100 ml of this enzyme solution containing 1.0–1.4 g of protein (A_{280}/A_{260} method) was applied to an hydroxylapatite column (4 × 65 cm) which had been equilibrated with 1 mM potassium phosphate buffer, pH 6.8. The column was developed with a linear gradient of potassium phosphate buffer, pH 6.8, varying from 1 to 500 mM in a total of 8 l. Fractions of 20 ml each were collected at a flow rate of $\sim$1 ml/min. The major protein peak (F1) from this column was dialyzed, lyophilized, and stored frozen in water or buffer at pH 7.4. Each preparation was checked for homogeneity using the Ornstein–Davis system of disc gel electrophoresis (Ornstein, 1964; Davis, 1964) using 7% gels and a running pH of 8.9. Only preparations which were homogeneous by this criterion were used in the studies reported here. Su *et al.* (1969) determined that fresh enzyme migrated as a single band in disc gel electrophoresis at various pH values and migrated as a single symmetric peak in the ultracentrifuge.

Assay of Enzymatic Activity. Method I was conversion of [7-^{14}C]nicotinamide to [7-^{14}C]nicotinic acid. This method was described by Kirchner *et al.* (1966) and Su *et al.* (1969). Method II was ammonia production (indophenol method) according to Leffler (1967) as adapted by Chaykin (1969). Method III was hydrolysis of *p*-nitrophenyl acetate. The rate of hydrolysis was followed by recording the increase in absorbance at 405 mμ due to the appearance of *p*-nitrophenolate ion at pH 7.4 (ϵ_{405} 1.29×10^4 M^{-1} cm^{-1}). The stock substrate solution was prepared daily. *p*-Nitrophenyl acetate was dissolved in 0.4 ml of acetonitrile and water was added to 25 ml to give a solution that was 2×10^{-3} M *p*-nitrophenyl acetate. Acetonitrile at the concentrations used in the assay (maximum usually 0.05%) had no effect on the enzyme. The standard assay mixture contained 6.7×10^{-5} M *p*-nitrophenyl acetate; 0.033 M potassium phosphate buffer, pH 7.4; and enzyme, all in a total of 3.0 ml.

Preparation of [^{32}P]DIP-protein. Nicotinamide deamidase (2.62×10^{-5} M) and [^{32}P]DFP (8.13×10^{-4} M) were incubated for 150 min at room temperature and then dialyzed against water at 4°. The sample was assayed, protein concentration was determined, and an aliquot was counted to determine moles of [^{32}P]DFP bound.

For the identification of the residue attacked by [^{32}P]DFP, the [^{32}P]DIP-protein was reduced, carboxymethylated, and cleaved with cyanogen bromide according to Anfinsen and Haber (1961), Craven *et al.* (1965), and Steers *et al.* (1965), and finally hydrolyzed in 6 N HCl under vacuum at 103° for 17 hr. The mild hydrolysis conditions described for [^{32}P]DFP-treated chymotrypsin (Schaffer *et al.*, 1953) were initially tried; however, chromatography and radioautography of that hydrolysate gave ambiguous results probably due to incomplete hydrolysis and incomplete separation of [^{32}P]P$_i$ and [^{32}P]serine phosphate. Hydrolysis in 6 N HCl resulted in a greater percentage of hydrolysis of the standard serine phosphate than with 2 N HCl. However, enough serine phosphate remained unhydrolyzed to be identified after chromatography. The hydrolyzed samples were subjected to descending paper chromatography in 77% ethanol for 15 hr followed by radioautography. Carrier serine phosphate and P$_i$ were added to the protein hydrolysate. After chromatography, the papers were sprayed with either 0.5% ninhydrin in acetone or a perchlorate–molybdate spray (Chaykin, 1966).

Reaction of Nicotinamide Deamidase with [^{14}C]ZPCK. A stock [^{14}C]ZPCK solution (5.97×10^{-3} M) was prepared in methanol. The reaction mixture contained 2.46×10^{-5} M protein; 0.1 M potassium phosphate buffer, pH 7.4; and 1.75×10^{-4} M [^{14}C]ZPCK and was 2.9% in methanol. The samples were incubated at 33° for 2.5 hr followed by exhaustive dialysis against water. Aliquots of the [^{14}C]ZPCK-protein were counted in a liquid scintillation counter to 3–5% error and assayed.

Reaction of Nicotinamide Deamidase with DTNB and [^{14}C]ZPCK. The reaction of nicotinamide deamidase with DTNB was carried out according to Ellman (1958). Since nicotinamide deamidase itself absorbs at 412 mμ, all absorbancy readings were corrected. The number of SH groups reacting with the reagent was calculated using an ϵ_{412} of 1.36×10^4 M^{-1} cm^{-1} for the anion released on reaction. At the end of the reaction the samples were dialyzed against 0.1 M potassium phosphate buffer, pH 7.4. The reaction of native enzyme and DTNB-enzyme with [^{14}C]ZPCK was carried out as described above with a 2-hr incubation at 37° followed by dialysis and analysis of activity.

COOH-Terminal Amino Acid Analysis. Digestion of nicotinamide deamidase with DFP-treated carboxypeptidase-A was performed as described by Ambler (1967). Samples were withdrawn at various times and were lyophilized to dryness and analyzed for free amino acids. The amounts of amino acids due to carboxypeptidase digestion of itself were negligible.

pH Optima. The radioactive amidase assay was used. The initial nicotinamide concentration was 0.137 M. The spectrophotometric esterase assay was used: reactions were followed by observation of *p*-nitrophenol at 340 mμ in the pH range 3.0–6.5 (Milstien and Fife, 1969) or of *p*-nitrophenolate ion at 405 mμ from pH 6.5 to 10.5. The initial *p*-nitrophenyl acetate concentration was 6.7×10^{-5} M. All rates were corrected for nonenzymatic hydrolysis of the substrates. Molar absorbancies of *p*-nitrophenol at 405 and 340 mμ were determined in the buffers used.

Inhibitor Studies. For amidase activity, the reaction mixtures contained 0.1 ml of inhibitor; 0.1 ml of enzyme in 0.1 M

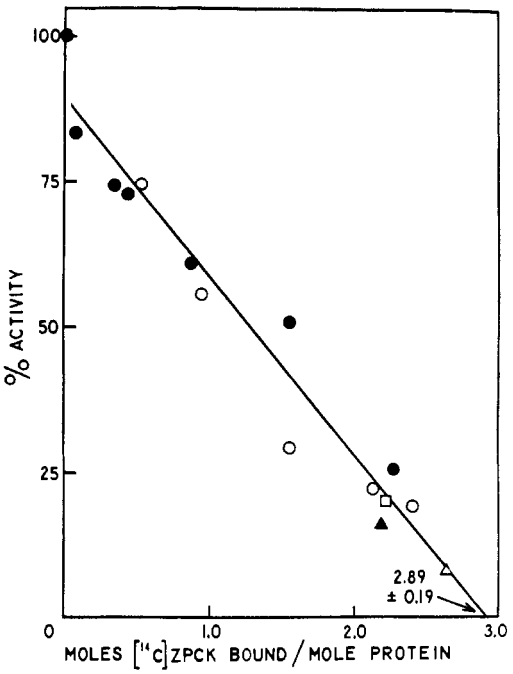

FIGURE 1: Variation of the ratio of [^{14}C]ZPCK to nicotinamide deamidase concentrations. Protein (2.31×10^{-5} M) in 0.05 M potassium phosphate buffer, pH 7.4, was incubated with [^{14}C]ZPCK ranging in concentration from 1×10^{-5} to 1.7×10^{-4} M at 37° for 105 min. The reaction mixtures were either separated on a Sephadex G-25 column and then dialyzed against 0.05 M potassium phosphate buffer, pH 7.4, or dialyzed without prior separation. The indophenol amidase assay was used and the samples were counted as described in the text. Different symbols are used for experiments run on different days. For each experiment the specific activity of the starting enzyme with no treatment was used as the 100% activity reference for those points.

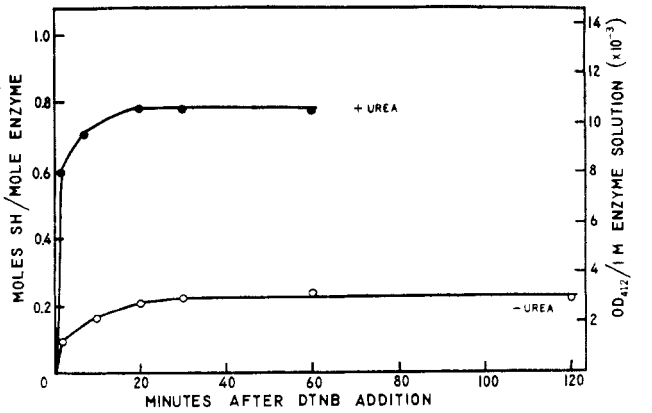

FIGURE 2. Time course of DTNB reaction with native and urea-treated nicotinamide deamidase. Experimental conditions were those of Ellman (1958). The reaction mixture with urea was 6 M in urea, pH 8.0.

potassium phosphate buffer, pH 7.4; and 0.1 ml of [7-^{14}C]-nicotinamide. Substrate concentrations in the reaction mixtures were 0.137 M (S_1) and 0.046 M (S_2).

For esterase activity, the standard inhibitor reaction mixture contained 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.4; 1.8 ml of inhibitor solution; 0.1 ml of enzyme solution; and 0.1 ml of *p*-nitrophenyl acetate substrate. *p*-Nitrophenyl acetate concentrations in the reaction mixtures were 6.7×10^{-5} M (S_1) and 3.3×10^{-5} M (S_2), except where noted otherwise.

Equilibrium Dialysis. A method similar to that of Rosenberg and Klotz (1960) was used. Dialysis tubing was prepared as described by Teipel and Hill (1968). The final soak was in 0.1 M potassium phosphate buffer, pH 7.4, overnight at 4°. All enzyme and ligand solutions were in the same buffer ($\Gamma/2 = 0.222$). No correction for the Donnan effect was made. Enzyme solution (1.0 ml) was equilibrated with *p*-nitrophenol solution (15 ml) and [^{3}H]H_2O (0.1 ml, 5.7×10^4 cpm/mmole). After 16 hr the solutions were analyzed for the ligand *p*-nitrophenol by measuring the A_{400}. An aliquot was counted in Bray's solution (Bray, 1960) in a liquid scintillation counter to allow determination of the total reaction volume. The protein concentration was determined using the biuret method with nicotinamide deamidase as the standard. The A_{400} of the protein–ligand solution was corrected for the absorption due to the protein at this wavelength ($A^{1\%}_{400}$ of the protein is ~0.080). The λ_{max} and ϵ of *p*-nitrophenol at pH 7.4 were unchanged in the presence of the protein. The ϵ_{400} of *p*-nitrophenol at pH 7.4 is 1.23×10^4 M^{-1} cm^{-1}. Control experiments indicated that equilibrium was established within 4 hr and that *p*-nitrophenol binding to the dialysis tubing was too small to be detected by this technique.

Results

Reaction of Nicotinamide Deamidase with [^{32}P]DFP. Reaction of nicotinamide deamidase with [^{32}P]DFP resulted in covalent binding of 2.79 moles of [^{32}P]DIP/mole of protein. Complete loss of amidase activity occurred. The specific esterase activity of a typical DIP-nicotinamide deamidase preparation was ~0.1% that of the starting enzyme. There was some loss (~10%) of amidase activity in the control enzyme sample incubated with 16% propylene glycol, the same percentage as that found in the [^{32}P]DFP-treated sample.

After hydrolysis of the [^{32}P]DIP-nicotinamide deamidase, two radioactive compounds were detected by radioautography; namely, serine phosphate and P_i, identified by co-chromatography and staining characteristics with ninhydrin and with perchlorate–molybdate.

Reaction of Nicotinamide Deamidase with [^{14}C]ZPCK. Reaction of nicotinamide deamidase with [^{14}C]ZPCK resulted in covalent binding of 2.63 moles of [^{14}C]ZPCK bound/mole of enzyme. The specific amidase activity of the ZPCK-enzyme was 0.40 μmole/mg hr, and of the control enzyme was 4.75 μmoles/mg hr: a 91.6% inhibition of amidase activity.

The results of a concentration study of the ZPCK reaction are shown in Figure 1. A computer best fit of the data produced a line which extrapolated to 2.89 ± 0.19 moles of [^{14}C]ZPCK bound per mole of protein at the point where no amidase activity remained.

Reaction of Nicotinamide Deamidase with DTNB and [^{14}C]ZPCK. Previous work had shown that treatment of rabbit liver nicotinamide deamidase with the SH reagents *N*-ethylmaleimide, *p*-chloromercuribenzoate, and iodoacetic acid had little effect on the activity of the enzyme (Su *et al.*, 1969), indicating that SH group(s) are not essential for activity. Further support for this and a concomitant indication that the [^{14}C]ZPCK reagent was probably attacking histidyl residues and not cysteinyl residues on the enzyme was sought in this study. The time course of the DTNB reaction with native and urea-treated nicotinamide deamidase is shown in Figure 2. After 1-hr incubation with DTNB 0.24 mole of reagent was bound per mole of native enzyme. The urea-treated enzyme bound 0.78 mole of reagent per mole of enzyme. The DTNB-

TABLE I: Effect of DTNB and [^{14}C]ZPCK Treatments on Nicotinamide Deamidase.

Enzyme Sample	Specific Activity (μmoles of N/mg hr)	Moles of DTNB Bound/ 211,000 g of Enzyme	Moles of [^{14}C]ZPCK Bound/ 211,000 g of Enzyme
Starting enzyme (No treatment)	3.87	0	0
Native enzyme + DTNB treatment	3.87	0.24	0
Native enzyme + 6 M urea + DTNB treatment	0.07	0.78	0
Native enzyme + [^{14}C]ZPCK treatment	0.65	0	2.15
Native enzyme + DTNB treatment then [^{14}C]ZPCK treatment	0.78	0.24	2.21

treated native enzyme sample, when incubated with [^{14}C]-ZPCK, bound 2.21 moles of [^{14}C]ZPCK per mole of enzyme with an 80% loss of amidase activity. An untreated enzyme sample in the same experiment bound 2.15 moles of [^{14}C]-ZPCK per mole of enzyme with an 83% loss of activity. These results are summarized in Table I.

COOH-Terminal Amino Acid Analysis. Leucine was the most rapidly released amino acid during carboxypeptidase-A digestion of nicotinamide deamidase. After 24 hr of digestion 0.90 mole of leucine per mole of deamidase was released. Other amino acids were released in lesser amounts.

pH Optima. At constant ionic strength there was a relatively broad peak of amidase activity from pH 6.5–8.5 (Figure 3). In this region the ionic strength of the potassium phosphate buffer had a small effect on the reaction. With this buffer the amidase activity was slightly higher at lower ionic strengths and the activity peaked at pH 7.5, the reported optimum pH (Kirchner *et al.*, 1966). Other than this, the ionic strength effect was within experimental error. Separate experiments indicated that from pH 5.0 to 10.0 the enzyme was stable under the reaction conditions of 1 hr at 37° at an enzyme

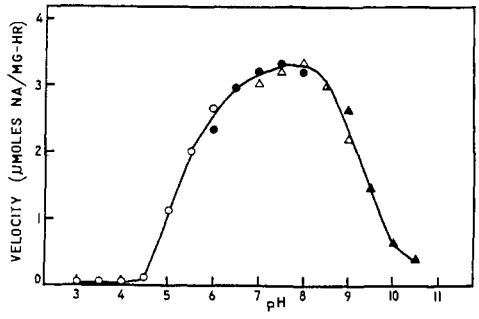

FIGURE 3: pH optimum of amidase activity at constant ionic strength. The buffers used were ○, malic acid; ●, potassium phosphate; △, Tris-HCl; ▲, glycine. The ionic strength of all buffers (except malic acid at pH 6.0) was adjusted to 0.272 with solid KCl. The ionic strength of the malic acid, pH 6.0, buffer was 0.280. NA = nicotinic acid.

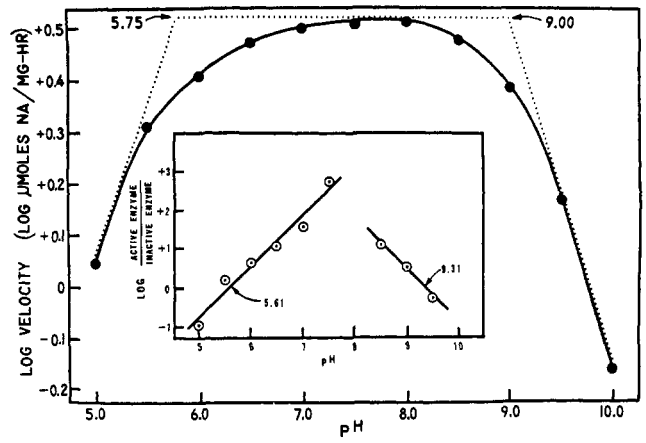

FIGURE 4: Plot of log v *vs.* pH of the data of Figure 3. The pK_a values of the ionizing groups in the ES complex affecting amidase activity are shown in the figure. Inset: Linear plot of the same data. The highest point on the log v curve was taken as the point of 100% active enzyme. The pK_a values of the ionizing groups determined from this plot are shown. NA = nicotinic acid.

concentration of 0.69 mg/ml. The pK_a values of the ionizing residues on the enzyme were estimated from a log v *vs.* pH plot of the data (Figure 4) (Dixon and Webb, 1964). A computer best fit of the data in a linear plot (Figure 4 inset) gave pK_a values for increasing and decreasing activity of 5.61 and 9.31, respectively.

Similar experiments performed with the ester substrate *p*-nitrophenylacetate at 6.7×10^{-5} M are shown in Figure 5. At constant ionic strength the pH optimum appeared to be in the region of pH 8.0–10.0. However, nonenzymatic hydrolysis of the substrate in this pH range is increasing (zero at pH 8.0 to 60% of the observed rate at pH 10.5) and thus the enzymatic rate is subject to a relatively larger error. Separate experiments showed that the enzyme was stable from pH 4.0 to 10.5 for the 3-min incubation period at a concentration of 2.7×10^{-4} mg/ml.

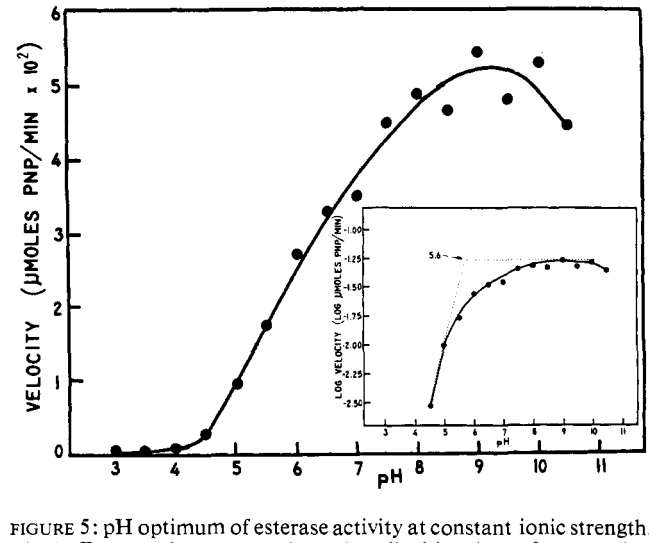

FIGURE 5: pH optimum of esterase activity at constant ionic strength. The buffers are the same as those described in Figure 3 except that sodium bicarbonate buffers ($\Gamma/2 = 0.272$) were used in place of glycine buffers. Average velocity values are reported at pH values where more than one kind of buffer was tested. Inset: Plot of log v *vs.* pH of the same data. See text for discussion of the pK_a value shown in the figure. PNP = *p*-nitrophenol.

TABLE II: K_i Values of Competitive Inhibitors.[a]

Inhibitor	K_i *vs.* Nicotinamide ± s.d. (M)	K_i *vs.* *p*-Nitrophenyl Acetate ± s.d. (M)
Nicotinic acid	$(1.16 \pm 0.30) \times 10^{-2}$	$(2.94 \pm 0.78) \times 10^{-2}$
p-Nitrophenol	$(3.71 \pm 0.25) \times 10^{-5}$	$(3.55 \pm 1.93) \times 10^{-5}$
Benzoic acid	$(5.70 \pm 0.45) \times 10^{-4}$	$(1.48 \pm 0.46) \times 10^{-3}$
Hydrocinnamic acid	$(1.26 \pm 0.08) \times 10^{-4}$	$(8.50 \pm 3.00) \times 10^{-4}$
3-Indolepropionic acid	$(1.06 \pm 0.14) \times 10^{-3}$	$(2.12 \pm 1.21) \times 10^{-3}$

[a] K_m of nicotinamide = $(2.74 \pm 0.06) \times 10^{-2}$ M. K_m of *p*-nitrophenyl acetate = $(4.06 \pm 0.19) \times 10^{-5}$ M.

Studies of the K_m and V_{max} of nicotinamide deamidase at different pH values indicated that the change in amidase activity with pH is due to a reversible effect of V_{max} and that affinity of the enzyme for nicotinamide as indicated by a constant K_m is not affected (Albizati, 1970).

Inhibitor Studies. All of the inhibitors studied (nicotinic acid, *p*-nitrophenol, benzoic acid, hydrocinnamic acid, and 3-indolepropionic acid) were competitive with both the amide and ester substrates at low inhibitor concentrations. The K_i values obtained from the straight-line portion of the curves are shown in Table II. At higher concentrations the velocity^{-1} *vs.* [I] plots of several inhibitors curved upward from a straight line. In these cases Hill plots were made and a computer best fit of the data was used to determine the slopes of the lines and $I_{0.5}$ values. The n values in all cases are close to 1.0 except in the case of nicotinamide inhibition of *p*-nitrophenyl acetate hydrolysis where the n value is 1.7, and in the case of *p*-nitrophenol inhibition of nicotinamide hydrolysis where the n value is 1.4, suggesting two or more weakly interacting sites (Table III).

Figure 6 shows that nicotinamide is bound to the ZPCK-enzyme and inhibits the esterolytic action of the modified enzyme even though it is not hydrolyzed itself. The apparent allosteric-type inhibitor kinetics observed with the native enzyme using *p*-nitrophenyl acetate as substrate and nicotinamide as inhibitor (Figure 7) are also exhibited by the ZPCK-enzyme (Figure 6 inset). The ZPCK-enzyme using *p*-nitrophenyl acetate in the absence of inhibitor exhibited hyperbolic kinetics with a $K_m = (4.96 \pm 0.40) \times 10^{-5}$ M and a $V_{max} = 5390$ μmoles/mg hr. These are in good agreement with the kinetic constants of the native enzyme for this substrate ($K_m = 4.06 \times 10^{-5}$ M, $V_{max} = 6300$ μmoles/mg hr). The production of sigmoidal kinetics by the addition of nicotinamide suggests a weak heterotropic interaction between the nicotinamide sites and the *p*-nitrophenyl acetate sites.

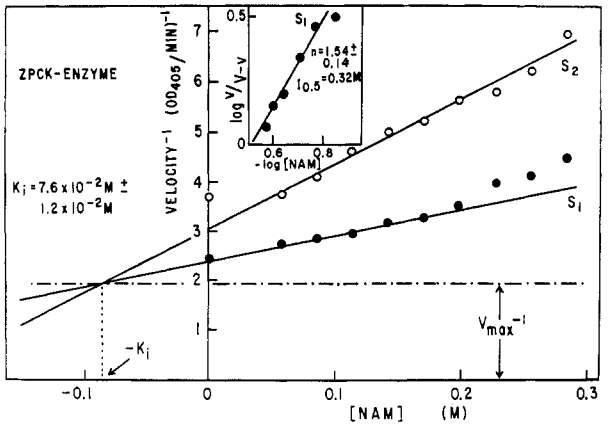

FIGURE 6: Nicotinamide (NAM) inhibition of ZPCK-enzyme hydrolysis of *p*-nitrophenyl acetate. Reaction conditions were those described in the text for the standard esterase assay with inhibitors. $S_1 = 1.47 \times 10^{-4}$ M. $S_2 = 7.33 \times 10^{-5}$ M. The K_i obtained from these data is shown in the figure. The control enzyme (non-ZPCK-treated) in this experiment gave a $K_i = 0.083 \pm 0.017$ M. The V_{max}^{-1} was determined using the Michaelis–Menten equation, $K_m = 4.06 \times 10^{-5}$ M, and $v = 0.409$ OD/min (v at zero nicotinamide, $S_1 = 1.47 \times 10^{-4}$ M). Inset: Hill plot of the data of S_1. The values of n and $I_{0.5}$ are shown in the figure. With S_2 the Hill plot slope is 1.31 ± 0.09.

Nicotinamide Saturation and the Effect of Fatty Acid. Figure 8 shows the results of an experiment in which the amidase activity was assayed in the presence and absence of 0.41 μg of palmitic acid. The slope of the Hill plot in the presence of palmitic acid is 1.35 ± 0.12. The control in this experiment gave a Hill plot slope of 1.00 ± 0.04.

Equilibrium Dialysis. The binding constant and number of *p*-nitrophenol binding sites on nicotinamide deamidase were determined using a Scatchard plot.[2] It was assumed that *p*-nitrophenol and nicotinic acid are each bound to the same site or the same binding center as their respective substrates.

The data from eight experiments using concentrations of *p*-nitrophenol from 0.7×10^{-5} to 11×10^{-5} M and enzyme from 1.02 to 4.83 mg/ml are shown in Figure 9. A computer best fit of the data from $\bar{\nu} = 0$ to $\bar{\nu} = 2.0$ produced a line with a slope of $(-4.25 \pm 0.52) \times 10^4$ M^{-1} which extrapolated to the abscissa at $\bar{\nu} = 3.03 \pm 0.42$. This slope can be expressed as the binding constant and is equal to the reciprocal of the dissociation constant, K_i, when the binding is described by the following equation, and $k_3 \ll k_2$, and without implying anything about the steps between ES and E + P.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

The K_i was determined kinetically to be $(3.55 \pm 1.93) \times 10^{-5}$ M with *p*-nitrophenyl acetate as substrate. The reciprocal of the binding constant, $(4.25 \pm 0.52) \times 10^4$ M^{-1}, is $(2.35 \pm 0.32) \times 10^{-5}$ M.

[2] The Scatchard equation in straight-line form is: $(\bar{\nu}/c)e^{2\bar{z}_p z_e \omega} = -k^0 \bar{\nu} + k^0 n$, in which n is the no. of binding sites of the same intrinsic affinity, k^0; $\bar{\nu}$ is the molar ratio of the bound ligand to protein; c is the molar concentration of the ligand; $\bar{z}_p$ and z_e are the net charges on the protein molecule and ligand molecule, respectively; and ω is the electrostatic parameter of the Debye–Huckel theory. The electrostatic term ($e^{2\bar{z}_p z_e \omega}$) equals 1 when the small molecule has zero net charge. A plot of $(\bar{\nu}/c)e^{2\bar{z}_p z_e \omega}$ *vs.* $\bar{\nu}$ gives a straight line with an ordinate intercept $k^0 n$, an abscissa intercept n, and a slope of $-k^0$. When more than one set of sites of intrinsic constant k_i^0 and number of sites n_i are present, a curved line is obtained.

TABLE III: $I_{0.5}$ and n Values from Hill Plots.[a]

Inhibitor	Amide Hydrolysis	
	S_1	S_2
Nicotinic acid	$n = 1.21 \pm 0.23$ $I_{0.5} = (7.62 \pm 3.81) \times 10^{-2}$ M	$n = 1.11 \pm 0.03$ $I_{0.5} = (3.28 \pm 0.35) \times 10^{-2}$ M
p-Nitrophenol	$n = 1.40 \pm 0.06$ $I_{0.5} = (2.26 \pm 0.88) \times 10^{-4}$ M	$n = 1.20 \pm 0.03$ $I_{0.5} = (9.49 \pm 2.07) \times 10^{-5}$ M
	Ester Hydrolysis	
	S_1	S_2
Nicotinic acid	$n = 1.19 \pm 0.06$ $I_{0.5} = (4.99 \pm 0.74) \times 10^{-2}$ M	
p-Nitrophenol	$n = 1.11 \pm 0.01$ $I_{0.5} = (1.17 \pm 0.09) \times 10^{-4}$ M	
Nicotinamide	$n = 1.67 \pm 0.08$ $I_{0.5} = (2.46 \pm 0.17) \times 10^{-1}$ M	$n = 1.57 \pm 0.09$ $I_{0.5} = (1.97 \pm 0.19) \times 10^{-1}$ M

[a] Nicotinamide substrate: $n = 1.06 \pm 0.05$, $S_{0.5} = (2.59 \pm 0.51) \times 10^{-2}$ M. *p*-Nitrophenyl acetate substrate: $n = 1.04 \pm 0.06$, $S_{0.5} = (3.98 \pm 2.93) \times 10^{-5}$ M.

The curved Scatchard plot indicates nonequivalent binding sites. In the case of nicotinamide deamidase, there are apparently three binding sites having similar affinities for *p*-nitrophenol. Nonspecific binding probably occurs at higher *p*-nitrophenol concentrations.

Competitive binding studies similar to those used with phosphofructokinase (Kemp and Krebs, 1967) and serum albumin (McMenamy and Oncley, 1958) were used to determine if the binding sites for the amide substrate are the same as the binding sites for the ester substrate. Figure 10 shows the competition between *p*-nitrophenol and 3 to 30 mM nicotinic acid. When 30 mM nicotinic acid was included in the binding solution, it effectively competed with *p*-nitrophenol over the concentration range studied producing a nearly horizontal line. Both 14 and 7 mM nicotinic acid competed at low *p*-nitrophenol concentrations but as the *p*-nitrophenol concentration was raised the competition was overcome and the curves began to slope downward as in a normal Scatchard plot. The $\bar{\nu}$ values obtained from extrapolation of these curves to the abscissa and the binding constants determined from the slopes of the computer-fitted lines are presented in Table IV.

The DFP-treated enzyme does not hydrolyze nicotinamide or *p*-nitrophenyl acetate. *p*-Nitrophenol ($1-5 \times 10^{-5}$ M) was

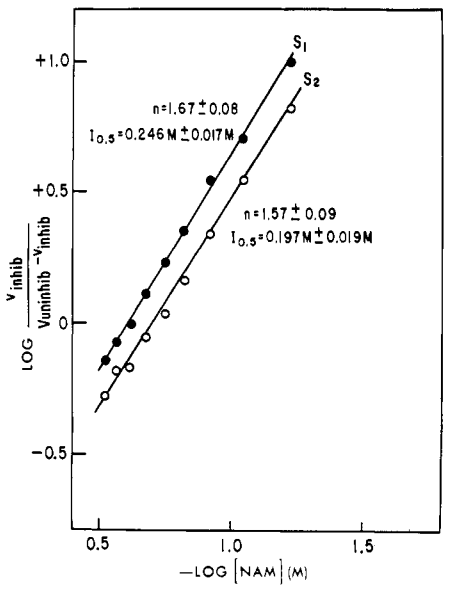

FIGURE 7: Hill plot of nicotinamide (NAM) inhibition of *p*-nitrophenyl acetate hydrolysis using native enzyme. See text for experimental conditions. The values of n and $I_{0.5}$ are shown.

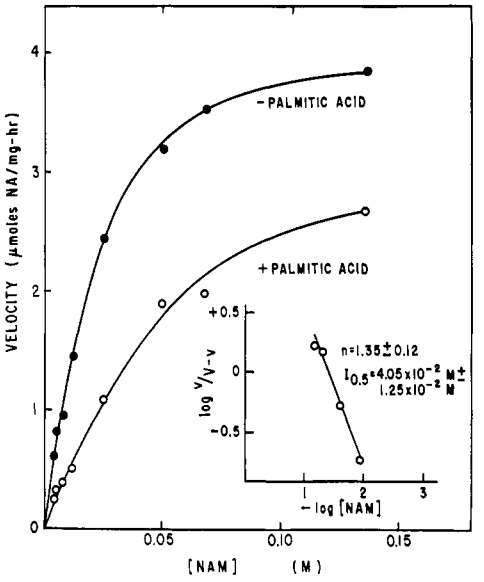

FIGURE 8: Velocity *vs.* [*S*] plot showing the effect of palmitic acid on the amidase activity of nicotinamide deamidase. Reaction mixtures contained 51 μg of enzyme; 0.033 M potassium phosphate buffer, pH 7.4; and substrate [7-^{14}C]nicotinamide (NAM) in a total volume of 0.3 ml. ●, control; ○, + 0.41 μg of palmitic acid. Inset: Hill plot of the data obtained with added palmitic acid. NA = nicotinic acid.

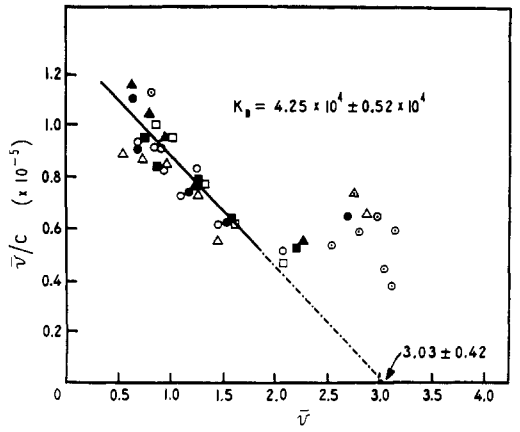

FIGURE 9: Scatchard plot of the binding of *p*-nitrophenol to nicotinamide deamidase. The equilibrium dialysis experiments are described in the text. The different symbols are used for experiments done on different days. The slope of the line, expressed as a binding constant, is shown in the figure.

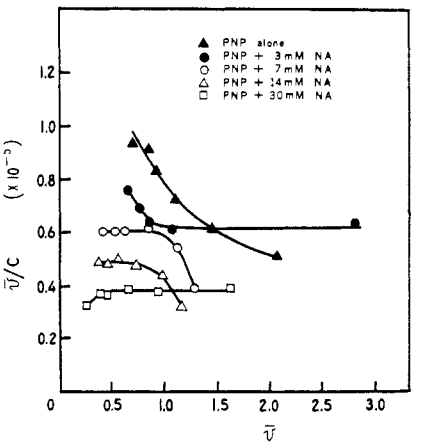

FIGURE 10: Scatchard plot of competitive binding studies. The binding of *p*-nitrophenol (PNP) alone and in the presence of varying concentrations of nicotinic acid (NA) is shown. See text for experimental procedure.

not bound to DIP-nicotinamide deamidase. Nicotinamide binding could not be studied.

Binding studies indicated that 1–5 $\times$ 10^{-5} M *p*-nitrophenol was bound to the ZPCK-protein from 10–20% as much as to native protein. Increases in *p*-nitrophenol concentration up to 5 $\times$ 10^{-4} M did not result in any change in the $\bar{\nu}/c$ value, 0.117 $\times$ 10^5 at 5 $\times$ 10^{-5} M *p*-nitrophenol ($\bar{\nu}$ = 0.577). The $\bar{\nu}/c$ value for native protein at $\bar{\nu}$ = 0.577 obtained from Figure 9 is 1.06 $\times$ 10^5. Competitive binding studies with ZPCK-protein, *p*-nitrophenol, and nicotinic acid showed that in the presence of nicotinic acid the binding of *p*-nitrophenol was reduced. The theoretical $\bar{\nu}/c$ for the native enzyme at $\bar{\nu}$ = 0.125 was 1.25 $\times$ 10^5. With the ZPCK-enzyme the $\bar{\nu}/c$ = 0.065 $\times$ 10^5 at nearly the same $\bar{\nu}$. In the presence of 0.03 M nicotinic acid the $\bar{\nu}/c$ at $\bar{\nu}$ = 0.124 was reduced to 0.026 $\times$ 10^5, suggesting that nicotinic acid was bound to the ZPCK-enzyme in agreement with the kinetic results. These results are summarized in Table V.

Discussion and Conclusions

Evidence That Ester and Amide Substrates Bind at the Same Sites on the Enzyme. Kinetic and binding experiments indicate that the two kinds of substrate of nicotinamide deamidase have the same enzyme binding sites. The inhibitors studied are all competitive with respect to both substrates. Each inhibitor exhibits the same, or nearly the same, K_i with both the amide and ester substrates. Binding experiments show that nicotinic acid at 0.03 M ($\sim K_i$) effectively competes with *p*-nitrophenol for the three primary binding sites on the native enzyme and on the ZPCK-enzyme. The results at lower nicotinic acid concentrations suggest that *p*-nitrophenol binds more strongly to two of the sites, or nicotinic acid binds more strongly at the third site. The nicotinic acid binding at two sites, but not at the third site, can be overcome by higher *p*-nitrophenol concentrations (Figure 10). From these experiments it is concluded that the amide and ester substrates bind at the same three sites although the binding of one of the substrates may not be equal at all three sites.

Evidence That the Ester and Amide Substrates Have Overlapping Catalytic Sites and/or Are Hydrolyzed by Different Mechanisms. Until recently the mechanism and active center of enzymes having a dual activity were thought to be identical for both activities. This was true in the case of several enzymes which exhibit proteolytic (amidolytic) and esteratic activities (Cunningham, 1965).

Recently, however, several research groups examining the activities of carboxypeptidase-A, trypsin, and α-chymotrypsin have obtained results indicating that these enzymes have nonidentical active centers or binding sites or different mechanisms for the two activities. Work on carboxypeptidase-A has revealed a complex catalytic system involving a metal, tyrosyl

TABLE IV: Binding Constants of *p*-Nitrophenol in the Presence and Absence of Nicotinic Acid.

Ligand	$\bar{\nu}$	Binding Constant of *p*-Nitrophenol (M^{-1})
p-Nitrophenol	3.03	(4.25 ± 0.52) $\times$ 10^4
p-Nitrophenol + 3 mM nicotinic acid	1.82	(6.58 ± 0.14) $\times$ 10^4
p-Nitrophenol + 7 mM nicotinic acid	2.16	(4.82 ± 1.82) $\times$ 10^4
p-Nitrophenol + 14 mM nicotinic acid	2.13	(3.56 ± 1.26) $\times$ 10^4

TABLE V: Binding of *p*-Nitrophenol to ZPCK-Enzyme and the Effect of Nicotinic Acid.

Enzyme	Starting *p*-Nitrophenol (M)	Nicotinic Acid (M)	$\bar{\nu}$	$\bar{\nu}/c$ ($\times$ 10^{-5})
Native enzyme	<0.7 $\times$ 10^{-5} [a]	0	0.125	1.25[b]
ZPCK-enzyme	2.42 $\times$ 10^{-5}	0	0.137	0.065
ZPCK-enzyme	5.27 $\times$ 10^{-5}	0.03	0.124	0.026

[a] The lowest value of [*p*-nitrophenol] actually used in experiments was 0.7 $\times$ 10^{-5} M. The $\bar{\nu}$ in that case was 0.62. A $\bar{\nu}$ of 0.125 estimated from Figure 9 would be obtained at a very low *p*-nitrophenol concentration. [b] This value is estimated from Figure 9.

residues, and varying degrees of activation and inhibition of the two activities under different conditions. A recent review (Blow and Steitz, 1970) suggests that ester and peptide substrates bind similarly to the enzyme but the hydrolysis of some esters occurs by a mechanism different from that of peptide hydrolysis. Coletti-Previero *et al.* (1969) reported that formylation of several tryptophanyl residues in trypsin destroys peptidase activity while only slightly affecting esterase activity. Studies of α-chymotrypsin by Epand (1969) revealed that formation of the acyl enzyme intermediate in amide hydrolysis is not obligatory, thereby presenting a mechanistic difference in the handling of the two classes of substrate by the enzyme.

It is thus not surprising that the results here are consistent with the hypothesis that (a) the mechanisms of hydrolysis of amides and esters by nicotinamide deamidase are not identical and/or (b) the active centers of amide hydrolysis are not identical with those of ester hydrolysis but are probably overlapping. The evidence is threefold. (a) Covalent binding of 2.8 moles of [^{32}P]DFP per mole of protein completely inhibits both activities. This suggests that the esterase and amidase activities share a seryl residue in the transforming part of each of three active centers on the enzyme molecule. However, if [^{32}P]DFP were attacking tyrosine in addition to serine, the DIP-tyrosine formed would most likely be degraded completely to tyrosine and P_i under the conditions used (Murachi *et al.*, 1965). Therefore, while it is valid to say that the [^{32}P]DFP attacks seryl residues in nicotinamide deamidase we cannot say that this is the only type of residue attacked. (b) Variation of the ratio of [^{14}C]ZPCK to nicotinamide deamidase concentrations indicates that covalent binding of 3.0 moles of [^{14}C]ZPCK destroys amidase activity while leaving esterase activity and amide binding intact. Thus the amidase mechanism requires histidyl residues, the esterase mechanism does not. (c) The two activities exhibit slightly different pH curves. The esterase optimum is in a higher pH range than the amidase optimum. A group on the enzyme–substrate complex having a pK_a value of 5.6–5.8 (the histidyl residues) contributes to the amidase activity. The assignment of the pK_a for esterase activity is not clear since the conclusion that the observed pK_a value is due to an ionizing group on the ES complex is valid only if [S] $\gg K_m$ (*i.e.*, the enzyme is nearly all in the ES form). However, the limited solubility of *p*-nitrophenyl acetate in water precluded analysis at a high substrate concentration. Thus, the observed pK_a of 5.6 (Figure 5) is not necessarily the pK_a value of the group responsible for ester hydrolysis.

Although finding that *p*-nitrophenol is not bound to ZPCK-nicotinamide deamidase to the same degree as to the native enzyme appears inconsistent with the kinetic data, several explanations are possible.

(a) Although *p*-nitrophenol is bound to the modified enzyme to a lesser extent than to the native enzyme, the actual substrate is bound to the same extent on native and ZPCK-enzyme. This cannot be tested directly because the enzyme rapidly hydrolyzes *p*-nitrophenyl acetate making it unsuitable for use in binding studies. It is possible that covering up histidyl residues with ZPCK in the neighborhood of the binding site exposes a negatively charged residue which electrostatically repels *p*-nitrophenol but not *p*-nitrophenyl acetate. At pH 7.4 $\sim$ 60% of the *p*-nitrophenol is negatively charged (0^-) and $\sim$ 40% is in the OH form, based on a pK_a = 7.19 at 25° (Dictionary of Organic Compounds, 1965). At 5.46×10^{-5} M $\bar{\nu}_{\text{ZPCK-enz}}/\bar{\nu}_{\text{native enz}} = 0.20$, *i.e.*, with ZPCK-protein the moles of *p*-nitrophenol bound per mole of protein is 20% that of native protein. Using uncharged analogs such as *p*-nitroanisole, this hypotheses could be tested.

(b) Modification of the enzyme changes the rate-limiting step in ester hydrolysis such that the K_m of the modified enzyme does not even approach a binding constant as it does for native enzyme. This could also explain why the ZPCK-enzyme does not appear to hydrolyze nicotinamide under the usual assay conditions since its binding would be shifted to a higher region.

(c) The binding of *p*-nitrophenol to the same extent on ZPCK-enzyme as on the native enzyme requires the presence of substrate p-nitrophenyl acetate. This possibility cannot be tested for the reason mentioned in a.

Amidase activity most likely proceeds by the classical seryl-histidyl mechanism, since both seryl and histidyl residues are shown to be involved.

However, additional studies must be done before a mechanism for the ester hydrolysis can be proposed. The observed pK_a for ester hydrolysis may be due to a critical aspartyl or glutamyl residue, since it is apparently not due to histidyl residues. The participation of a metal or other cofactor also cannot be ruled out since Su and Chaykin (1971) have found that the deamidase is inhibited by 8-hydroxyquinoline and some other metal chelators. Furthermore, the deamidase has an absorption peak in the region of 400 mμ and the ratio of protein concentration to this absorption remains relatively constant from preparation to preparation, suggesting that it is due to a component of the protein. An average value of A_{215}/A_{405} for the pure enzyme from six preparations was 1140, ranging from 831 to 1420. Until these observations are more fully investigated, the possibility remains that a metal or other component is present and is contributing to one or both of the enzymatic activities.

The formation of an acyl enzyme intermediate in ester hydrolysis has been proposed based on the "burst" phenomenon observed in *p*-nitrophenyl nicotinate hydrolysis (see Figure 6 of Su *et al.*, 1969). That data was not unequivocal, however, since the apparent "burst" would disappear with a different extrapolation of the experimental points. The data presented here suggests, though again not unequivocally, that an acyl-enzyme intermediate is involved in ester hydrolysis.

Site Interaction. The product inhibition data indicate that weak interaction exists between sites. The addition of a substrate or product of the reaction not being measured (*i.e.*, addition of *p*-nitrophenol as inhibitor of amide hydrolysis, addition of nicotinic acid or nicotinamide as inhibitor of ester hydrolysis) gives an increase in the Hill interaction coefficient, *n*. The increase is greatest when the inhibitor is the substrate of the other reaction (*i.e.*, when nicotinamide inhibition of *p*-nitrophenyl acetate hydrolysis is studied). There is no increase in *n* when the inhibitor is the product of the reaction being studied. This suggests that a weak heterotropic interaction exists between the sites. This is not inconsistent with the hypothesis that the catalytic sites for the two activities are overlapping and the binding sites for the two substrates are the same. Changeux (1963) broadly defines allosteric proteins as having at least two distinct nonoverlapping receptor sites. The binding results here suggest that the receptor sites (binding sites) of nicotinamide deamidase are identical for both kinds of substrate, and there are at least two sites that have the same binding constants for the substrates. The allosteric effects observed here are, at best, weak, the largest interaction coefficient observed being 1.67 when nicotinamide is the effector (inhibitor) and *p*-nitrophenyl acetate is the substrate.

Su *et al.* (1969) reported that nicotinamide deamidase exhibits nonhyperbolic (sigmoidal) kinetics with the substrate nicotinamide at pH 7.5. Repeated attempts to reproduce this at pH 7.4 in this study were without success. The only anomaly observed in the kinetic results was nicotinamide inhibition at high concentrations (above $\sim$ 0.1 M). The Hill plot gives a straight line with a slope of 1.06 $\pm$ 0.05. These results indicate that with this preparation there is no homotropic allosteric effect with nicotinamide.

Known properties of the enzyme suggest two reasons for this discrepancy. (a) As the enzyme ages a conformational change destroys the interaction between sites. (b) The presence of traces of fatty acids or detergents which are not removed from the enzyme during purification or are later picked up from non-acid-washed glassware causes (or reduces) sigmoidal effects. Lipid material in crude deamidase preparations, some fatty acids, and detergents inhibit enzyme activity (Kirchner *et al.*, 1966; Su and Chaykin, 1971; Gillam *et al.*, 1972).

The enzyme assayed immediately after elution from the hydroxylapatite column did not show sigmoidal kinetics when assayed with either substrate. The Lineweaver–Burk plots were linear with nicotinamide (0.004–0.1 M) and with *p*-nitrophenyl acetate ($0.066–1.32 \times 10^{-4}$ M). Aged enzyme produced the same results.

The second hypothesis was tested by assaying the amidase activity of the enzyme in the presence of palmitic acid. The Hill plot slope with palmitic acid was greater than that of the control (Figure 8). Thus, contaminating lipid material may have caused the sigmoidal velocity *vs.* [S] curve previously obtained with nicotinamide. Although palmitic acid was used here, the lipid material thought to be associated with the crude enzyme or picked up by the enzyme during purification is probably a complex mixture of lipids.

Stoichiometry. The stoichiometry of *p*-nitrophenol binding and of ZPCK and DFP inhibition suggests that there are three active centers on the protein. The enzyme can be dissociated into four subunits (Su and Chaykin, 1971; Gillam *et al.*, 1972). Determination of COOH-terminal amino acids indicated that the most rapidly released amino acid was leucine, and after 24 hr of digestion with carboxypeptidase-A 0.90 mole of leucine was released per mole of deamidase. In addition, treatment of the enzyme in 6 M urea with DTNB gave reaction with 0.8 mole of SH per mole of protein.[3]

Thus the evidence presented here and elsewhere (Su *et al.*, 1969, Su and Chaykin, 1971) suggests that nicotinamide deamidase is composed of four subunits, three of which contain active centers and the fourth of which has the odd C-terminal leucine and SH group. This is possible, but not the most likely structure for a protein, since it is an unsymmetrical arrangement and few proteins with similar structures have been reported. Extensive studies of fructose 1,6-diphosphate aldolase have shown that it also has four subunits but apparently only three fructose diphosphate binding sites (Penhoet *et al.*, 1967). Penhoet *et al.* (1967) suggest that some of the substrate binding sites have different kinetic constants and have remained undetected. One other protein, *Neurospora* malic dehydrogenase, having a quaternary structure of the form $\alpha\alpha\alpha\beta$ has been reported (Munkres, 1968).

While the present findings are consistent with a tetrameric structure for nicotinamide deamidase with three active sites, further definitive investigations are needed to confirm this hypothesis.

[3] This value differs from that reported by Su *et al.* (1969).

Acknowledgments

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Multiple Pancreatic Lipases. Tissue Distribution and Pattern of Accumulation during Embryological Development†

William S. Bradshaw‡ and William J. Rutter*

ABSTRACT: Two lipases, designated A and B, have been detected in extracts of rat pancreas and in pancreatic juice. These enzymes can be distinguished from each other and from other esterolytic enzymes on the basis of their electrophoretic mobility and chromatographic properties as well as their substrate specificity and the differential effects of sodium taurocholate on their activity. Significant levels of lipases A and B are apparently found only in the pancreas and pancreatic juice; these enzymes were not detected in a number of other embryonic and adult tissues. Total lipase activity was measured in rudiments obtained from developing rat and mouse embryos and from postnatal and adult animals. A low but significant level of lipase A activity was found in the earlier stages of development of the rat pancreas (12 to 14 days gestation). This level was significantly higher than that present in other tissues of the embryo and, hence, is considered to be developmentally significant. The lipase specific activity of isolated rudiments increases dramatically (over 1000-fold) in embryos from 15 to 18 days gestation reaching a second plateau of activity characteristic of the late embryonic and newborn pancreases. The quantitative and temporal aspects of this pattern of development were duplicated in 13-day embryonic rat pancreases cultured *in vitro*. Lipase B was first detectable in the 18-day embryonic rat pancreas; the level slowly increases until birth at which time lipase B represents approximately 10% of the total lipase activity. Subsequently, however, the relative level increases substantially, for in the adult animal, lipase B contributes more than 50% of the total activity found in pancreatic extracts. The biphasic developmental pattern of lipase specific activity is similar to that found for a number of other specific exocrine proteins during pancreatic development and supports the concept of a biphasic process of differentiation in the exocrine cells.

Pancreatic lipase (EC 3.1.1.3, glycerol-ester hydrolyase) is one of the specific exocrine enzymes secreted by the pancreas in the pancreatic juice. Studies on purified pancreatic lipase from hog (Marchis-Mouren *et al.*, 1959; Entressangles *et al.*, 1966) indicate that the enzyme specifically hydrolyzes the primary ester bonds of emulsified triglycerides of long-chain fatty acids. The purified hog enzyme has been shown to be electrophoretically and chromatographically homogeneous (Marchis-Mouren *et al.*, 1959) and only a single lipase was detectable in rat pancreas by means of immunoelectrophoresis (Pascale *et al.*, 1966). However, a partially purified hog lipase has been resolved by Sephadex chromatography into two active components of significantly different molecular weight (Sarda *et al.*, 1964). The conversion of the "rapid" lipase to the "slow" form by methanol–ether extraction suggested that the former was a lipid-bound derivative of the latter.

We report here the results of studies aimed at determining (1) the number of distinct proteins contributing to pancreatic lipase activity; (2) the distribution of these lipases in other adult or embryonic tissues; (3) the patterns of accumulation of lipase activity in the developing pancreas *in vivo* and *in vitro*.

The developmental profiles of lipase activity emphasize

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‡ Present address: Department of Zoology, Brigham Young University, Provo, Utah 84801.

* Present address: Department of Biochemistry and Biophysics, University of California, San Francisco Medical Center, San Francisco, Calif. 94122; to whom correspondence should be addressed.