Useful relationships from these definitions are

$$\frac{V_{max,f}}{K_{m,H_2}} = \frac{V_{max,r}}{K_{i,H_2}} = \frac{k_1}{k_4}$$

and

$$K_{m,F_0}K_{i,H_2} = K_{m,H_2}K_{i,F_0}$$

The velocity equation can be written for the forward direction as a function of $[H_2]$ or $[F_0]$:

$$1/v = \frac{1}{[H_2]}\frac{K_{m,H_2}}{V_{max}}\left(1 + \frac{K_{i,F_0}}{[F_0]} + \frac{K_{i,F_0}[F_0H_2]}{[F_0]}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,F_0}}{[F_0]} + \frac{K_{m,F_0}}{K_{i,F_0}}\frac{[F_0H_2]}{[F_0]}\right)$$

$$1/v = \frac{1}{[F_0]}\frac{K_{m,F_0}}{V_{max}}\left(1 + \frac{[F_0H_2]}{K_{i,F_0}} + \frac{K_{i,H_2}}{[H_2]} + \frac{K_{m,F_0H_2}K_{i,H_2}}{K_{i,F_0H_2}[H_2]}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,H_2}}{[H_2]}\right)$$

In the absence of product ($F_0H_2$), the preceding equations predict intersecting initial velocity patterns. The predicted product inhibition patterns are noncompetitive vs. $[H_2]$ and competitive vs. $[F_0]$.

The fractional occupancy factors in the presence of a competitive inhibitor (I) for $H_2$ (e.g., CO) are modified:

$$f_1' = \frac{[H_2]/K_4}{1 + [H_2]/K_4 + [I]/K_I} = \frac{K_I[H_2]}{K_IK_4 + [H_2]K_I + K_4K_I} = \frac{K_I[H_2]}{d_1'}$$

$$f_{-1}' = \frac{1}{1 + [H_2]/K_4 + [I]/K_I} = \frac{K_I}{K_IK_4 + [H_2]K_I + K_4[I]} = \frac{K_I}{d_1'}$$

where

$$d_1' = K_IK_4 + [H_2]K_I + K_4K_I$$

The velocity equation is derived with $f_1'$ and $f_{-1}$. The reciprocal forms are as follows:

$$1/v = \frac{1}{[H_2]}\frac{K_{m,H_2}}{V_{max}}\left(1 + \frac{K_{i,F_0}}{[F_0]} + \frac{[I]}{K_i}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,F_0}}{[F_0]}\right)$$

$$1/v = \frac{1}{[F_0]}\frac{K_{m,H_2}}{V_{max}}\left(1 + \frac{K_{m,F_0H_2}K_{i,H_2}}{K_{i,F_0H_2}[H_2]}\right) + \frac{1}{V_{max}}\left(1 + \frac{K_{m,H_2}}{[H_2]} + \frac{K_{m,H_2}}{[H_2]}\frac{[I]}{K_i}\right)$$

A competitive inhibitor of $[H_2]$ is predicted to be uncompetitive vs. $F_0$ (in the presence of a constant amount of $[H_2]$).

# Evidence for an Essential Histidine in Neutral Endopeptidase 24.11[†]

Robert C. Bateman, Jr.,[*,‡] and Louis B. Hersh

*Department of Biochemistry, University of Texas Health Sciences Center at Dallas, Dallas, Texas 75235*

*Received November 3, 1986; Revised Manuscript Received March 11, 1987*

ABSTRACT: Rat kidney neutral endopeptidase 24.11, "enkephalinase", was rapidly inactivated by diethyl pyrocarbonate under mildly acidic conditions. The pH dependence of inactivation revealed the modification of an essential residue with a $pK_a$ of 6.1. The reaction of the unprotonated group with diethyl pyrocarbonate exhibited a second-order rate constant of 11.6 $M^{-1}$ $s^{-1}$ and was accompanied by an increase in absorbance at 240 nm. Treatment of the inactivated enzyme with 50 mM hydroxylamine completely restored enzyme activity. These findings indicate histidine modification by diethyl pyrocarbonate. Comparison of the rate of inactivation with the increase in absorbance at 240 nm revealed a single histidine residue essential for catalysis. The presence of this histidine at the active site was indicated by (a) the protection of enzyme from inactivation provided by substrate and (b) the protection by the specific inhibitor phosphoramidon of one histidine residue from modification as determined spectrally. The dependence of the kinetic parameter $V_{max}/K_m$ upon pH revealed two essential residues with $pK_a$ values of 5.9 and 7.3. It is proposed that the residue having a kinetic $pK_a$ of 5.9 is the histidine modified by diethyl pyrocarbonate and that this residue participates in general acid/base catalysis during substrate hydrolysis by neutral endopeptidase 24.11.

Neutral endopeptidase 24.11 (NEP, "enkephalinase", EC 3.4.24.11) is a mammalian membrane-bound zinc metalloendopeptidase that was first described by Kerr and Kenny (1974a,b). The enzyme has been purified to homogeneity from a number of sources (Kerr & Kenny, 1974a; Orlowski & Wilk, 1981; Gafford et al., 1983; Almenoff & Orlowski, 1983) and its substrate specificity studied in several laboratories (Turner et al., 1985; Pozsgay et al., 1986; Hersh & Morihara, 1986). NEP resembles the bacterial neutral metalloproteases in that it cleaves peptide bonds on the amino side of hydrophobic residues. However, the enzyme also displays a preference (but not absolute requirement) for a substrate containing a free COOH-terminal carboxylate, cleaving these substrates preferentially near their COOH terminus (Hersh & Morihara, 1986).

[†] Supported in part by National Institute on Drug Abuse Grant DA 02243 (to L.B.H.) and Welch Foundation Grant I391 (to L.B.H.).

* Address correspondence to this author.

[‡] Recipient of a Robert A. Welch postdoctoral fellowship.

0006-2960/87/0426-4237$01.50/0 

This endopeptidase has received considerable renewed interest since it has been shown to be identical with the brain enzyme given the trivial name "enkephalinase", an enzyme implicated in the degradation of endogenously released enkephalins in brain (Hersh, 1982; Matsas et al., 1983). In addition to the enkephalins (Hersh, 1984), NEP has been shown to degrade several biologically active peptides in vitro, including substance P (Matsas et al., 1983), bradykinin (Almenoff & Orlowski, 1983), and the chemotactic peptide (Connelly et al., 1985) to name but a few.

Despite the apparent importance of this enzyme in metabolism of bioactive peptides, its catalytic mechanism has not been studied in detail. In order to understand the enzymatic mechanism of, and possibly design mechanism-based inhibitors to NEP, it is helpful to identify active site amino acid residues participating in catalysis. Previously, the only residue identified at the active site of NEP is arginine, a residue thought to be involved in substrate binding (Malfroy & Schwartz, 1982; Jackson & Hersh, 1986). In this paper, we report the identification of an essential histidine residue at the active site of rat kidney neutral endopeptidase 24.11.

## Materials and Methods

Glutaryl-Gly-Gly-Phe-4MeO2NA[1] and glutaryl-Ala-Ala-Phe-4MeO2NA were from Enzyme Systems Products. Dansyl-D-Ala-Gly-Phe($NO_2$)-Gly (DAGNPG) was obtained from Calbiochem, and diethyl pyrocarbonate (DEPC) was obtained from Sigma Chemical Co. DEPC solutions were freshly prepared in absolute ethanol. The concentration was determined by addition of aliquots to a 10 mM imidazole solution, pH 7.5, and measurement of the increase in absorbance at 238 nM. A molar extinction coefficient of 3000 was used to calculate concentrations (Melchior & Fahrney, 1970).

Neutral endopeptidase 24.11 was solubilized from rat kidney membranes with papain and purified by a modification of the method of Almenoff and Orlowski (1983) as previously described (Jackson & Hersh, 1986). The purified enzyme migrated as a single band with a molecular weight of 90 000 on SDS–PAGE. This molecular weight was used as the subunit molecular weight.

Enzyme was routinely assayed at 23 °C with the substrate dansyl-D-Ala-Gly-Phe($NO_2$)-Gly (DAGNPG) by a continuous fluorometric assay procedure (Florentin et al., 1984). In this assay, 10 μL of an appropriately diluted enzyme solution was added to 240 μL of DAGNPG, 0.1 mM, in 0.05 M MES, pH 6.4, and the initial velocity measured by the increase in fluorescence at an excitation wavelength of 342 nm and an emission wavelength of 562 nm.

Kinetic constants were determined with glutaryl-Ala-Ala-Phe-4MeO2NA as substrate by a stopped assay procedure (Hersh & Morihara, 1986). In this assay, 10 μL of an appropriately diluted enzyme solution was added to 240 μL of glutaryl-Ala-Ala-Phe-4MeO2NA, 0.1 mM, in 0.05 M MES, pH 6.4, and incubated for 15 min at 37 °C. Ten microliters of a phosphoramidon/aminopeptidase M solution (40 μM phosphoramidon/0.25 mg/mL aminopeptidase M) was added, the mixture incubated for an additional 20 min at 37 °C, and the fluorescence measured at an excitation wavelength of 340 nm and an emission wavelength of 425 nm.

Protein was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

[1] Abbreviations: 4MeO2NA, 4-methoxy-2-naphthylamide; DAGNPG, dansyl-D-alanylglycyl-*p*-nitrophenylalanylglycine; DEPC, diethyl pyrocarbonate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

*Inactivation with DEPC.* NEP was diluted into MES buffer, 0.05 M, containing 0.2 M NaCl, to a final volume of 150 μL and a final protein concentration of 10 μg/mL. To this was added 0.5–4 μL of an ethanolic solution of DEPC (25–150 mM). This ethanol concentration had no effect on enzyme activity or stability. After incubation for various periods of time at 23 °C, aliquots (5–10 μL) were removed to measure remaining enzymatic activity by the DAGNPG method. The pseudo-first-order rate constant for inactivation was calculated by the initial rates method described by Neurath and co-workers (Burstein et al., 1974). Each determination was performed at least 3 times and was completed within 3 min. Control experiments showed insignificant nonenzymatic hydrolysis of diethyl pyrocarbonate under these conditions. Half-times of DEPC hydrolysis were measured as 20 min at pH 7 and 29 min at pH 6 in 0.05 M MES buffer containing 0.2 M NaCl. These values are consistent with the relatively long half-time of DEPC in MES buffer (28 min at pH 7) reported previously (Meyer & Cromartie, 1980).

In reactivation experiments, DEPC-modified NEP (<5% original activity) was made 50 mM in hydroxylamine by addition of 2 M hydroxylamine, pH 7.0, and assayed for activity after 1 and 2 h, and the activity was compared to controls of identically treated native enzyme. The concentration of hydroxylamine used did not affect enzyme stability or interfere with the assay.

To measure stoichiometry of modification by DEPC, 0.425 mL of NEP (0.33 mg/mL) in 0.05 M MES, pH 5.8, containing 0.2 M NaCl, was mixed with 6.5 μL of DEPC (20 mM) and the increase in absorbance at 240 nm monitored continuously over a time range in which enzyme activity was reduced to 10% of its original activity. The moles of histidine modified were calculated with a molar extinction coefficient of 3600 for *N*-carbethoxyhistidine (Miles, 1977). An identical enzyme solution was treated with DEPC in the same manner, and 1–5-μL aliquots were removed at various times to assay for enzyme activity with the DAGNPG substrate.

A similar spectral measurement of histidine modification was repeated in the presence of 35 μM phosphoramidon (molar ratio of phosphoramidon to enzyme = 10) after preincubation with this inhibitor for 10 min. Phosphoramidon was chosen for this experiment not only because it binds tightly to the active site at low concentrations ($K_i$ = 3 nM) but because it lacks the free amines of peptide substrates, which would react with DEPC and rapidly reduce its concentration. It also lacks the strong chromophores of the fluorogenic substrates, which would interfere with measuring changes in modified enzyme residues in the ultraviolet spectrum.

*Kinetic Measurements.* The kinetic constants of the partially DEPC-modified and native enzyme were determined by incubating 7.5 μg of NEP with 0.7 mM DEPC for 10 min, centrifuging the incubation mixture through a Bio-Gel P-6DG desalting column, and assaying the identically treated native and DEPC-modified enzymes with the very sensitive glutaryl-Ala-Ala-Phe-4MeO2NA substrate over the concentration range of 0.2–0.025 mM.

The dependence of $V_{max}/K_m$ on pH was determined with 10 μM DAGNPG, a concentration that is well below the $K_m$ value of 73 μM (Hersh & Morihara, 1986). Buffers used were MES (pH 5.2–7.2) and Tris-HCl (pH 7.1–9.1). Buffers were used at a concentration of 0.05 M and contained 0.2 M NaCl.

## Results

Treatment of rat kidney neutral endopeptidase 24.11 with 0.1–3 mM diethyl pyrocarbonate led to a rapid loss of enzymatic activity. Complete inactivation could be observed within

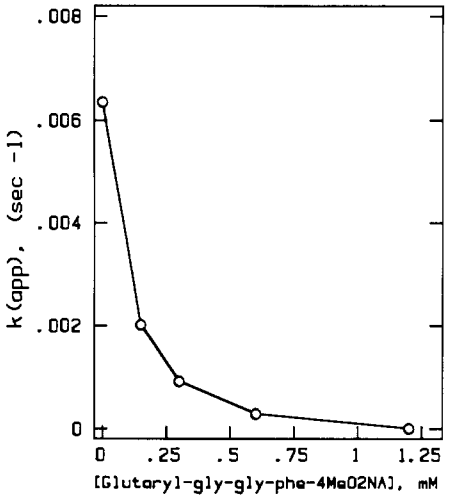


FIGURE 1: Effect of various concentrations of the substrate glutaryl-Gly-Gly-Phe-4MeO2NA on the rate of inactivation of neutral endopeptidase 24.11 (NEP) by diethyl pyrocarbonate (DEPC). Reactions were carried out at 23 °C in 0.1 M MES, pH 6.4, and contained 10 μg/mL NEP, 0.3 mM DEPC, and the indicated concentrations of glutaryl-Gly-Gly-Phe-4MeO2NA. The pseudo-first-order rate constant of NEP inactivation by DEPC is defined as $k_{app}$.

15 min at 1 mM DEPC. At high DEPC concentrations, enzyme inactivation was pseudo first order for at least four half-times. However, at low DEPC concentrations where the rate of DEPC hydrolysis was significant in comparison to the rate of enzyme inactivation, deviation from pseudo-first-order kinetics was apparent after approximately two half-times. Thus, rates of enzyme inactivation were calculated from initial rate measurements (Burstein et al., 1974). The rates of inactivation measured in this way were found to be proportional to the DEPC concentration.

The enzyme could be protected from inactivation by DEPC by inclusion in the inactivation reaction of glutaryl-Gly-Gly-Phe-4MeO2NA, a substrate with relatively high affinity ($K_m$ = 0.09 mM) but low turnover ($k_{cat}$ = 0.14 $s^{-1}$) (Hersh & Morihara, 1986). As shown in Figure 1, this substrate showed complete protection in a manner consistent with saturation of the active site. A plot of the half-time of inactivation against the concentration of glutaryl-Gly-Gly-Phe-4MeO2NA gives a dissociation constant of 0.07 mM, a number very close to the kinetic $K_m$. This result indicates that DEPC inactivation is due to reaction of a group at the active site of the enzyme.

*Evidence for Histidine Modification by DEPC.* To determine the type of residue modified by DEPC, the change in protein absorbance in the ultraviolet region upon DEPC modification was determined. As shown in Figure 2, the difference spectrum between enzyme alone and enzyme plus DEPC shows an increase in absorbance at 242 nm, a change characteristic of histidine modification (Miles, 1977). As can also be seen in Figure 2, reaction of the enzyme with DEPC does not result in a change in the absorbance at 280 nm, seemingly excluding tyrosine modification. Reaction of tyrosyl residues with DEPC generally results in a decrease in absorption at this wavelength (Muhlrad et al., 1967). Furthermore, acetylimidazole, a tyrosine-modifying reagent, has no effect on NEP activity when incubated with NEP for 1 h at 5 mM concentration. This concentration of acetylimidazole rapidly inactivates enzymes with reactive tyrosine groups, such as carboxypeptidase A (Auld & Vallee, 1970; Vallee & Riordan, 1968) and angiotensin-converting enzyme (Bunning et al., 1978).

The $pK_a$ of the reactive group was determined by plotting the reciprocal of the second-order rate constant of inactivation

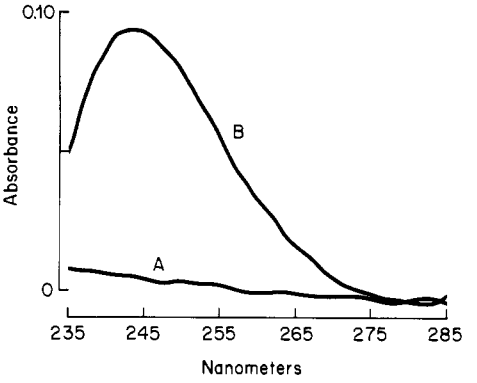


FIGURE 2: Difference absorbance spectrum of NEP before (A) and after (B) inactivation with DEPC. Samples consisted of 0.425 mL of enzyme (0.5 mg/mL) in 0.05 M MES, pH 6.4. DEPC was added to a concentration of 0.1 mM, and the spectrum was recorded after 20 min of incubation at 23 °C.

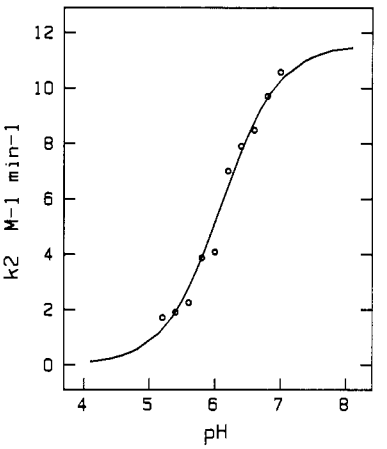


FIGURE 3: Effect of pH on the second-order rate of inactivation of NEP by DEPC. Conditions were as in Figure 1, except that 0.05 M MES buffer containing 0.2 M NaCl was used. The second-order rate constants ($k_2$) were calculated by dividing the pseudo-first-order rate constant for inactivation by the concentration of DEPC used (0.3 mM). The theoretical curve drawn assumes a $pK_a$ of 6.1.

against the hydrogen ion concentration as shown in Figure 3. For NEP at 23 °C, this plot reveals a $pK_a$ of 6.1 and an intrinsic second-order rate constant of 11.4 $M^{-1}$ $s^{-1}$. This second-order rate constant is comparable to those for DEPC modification of histidine residues in ribulosebisphosphate carboxylase/oxygenase (14 $M^{-1}$ $s^{-1}$) (Saluja & McFadden, 1982), L-α-hydroxyacid oxidase (11.5 $M^{-1}$ $s^{-1}$) (Meyer & Cromartie, 1980), luciferase (12 $M^{-1}$ $s^{-1}$) (Cousineau & Meighen, 1976), and *N*-acetylhistidine (20 $M^{-1}$ $s^{-1}$) (Holbrook & Ingram, 1973).

Although DEPC inactivated enzyme could not be reactivated by dilution, it was completely and rapidly reactivated by hydroxylamine at neutral pH. Within 1 h at 23 °C, 50 mM hydroxylamine reversed the inactivation by 90%, and complete activity was restored after 2 h.

*Stoichiometry of Modification.* Neutral endopeptidase 24.11 contains approximately eight histidine residues per subunit.[2] Of these eight residues, approximately three react rapidly with DEPC at pH 5.8, leading to a loss in enzyme activity (Figure 4). Although these three histidine residues all react rapidly with DEPC, they are not necessarily all essential for enzyme activity. Tsou (1962) demonstrated that the number of essential residues modified under conditions in

[2] T. A. Vida and L. B. Hersh, unpublished results.

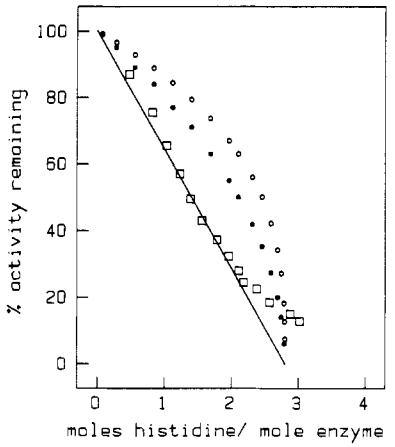


FIGURE 4: Correlation between enzyme activity and histidine residues modified by DEPC. Samples consisted of 0.425 mL of enzyme (0.33 mg/mL) in 0.05 M MES, pH 5.8, containing 0.2 M NaCl. DEPC was added to a final concentration of 0.3 mM, and the number of histidine residues modified was determined spectrophotometrically at 240 nm with an extinction coefficient of 3600. Theoretical lines drawn assume three residues modified under these conditions, with one (solid line), two (solid circles), or three (open circles) residues essential for activity. The squares are actual data points.

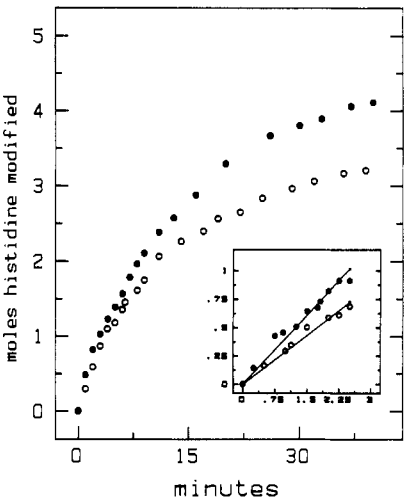


FIGURE 5: Extended time course of total histidines modified by DEPC. Conditions were as in Figure 4, except that phosphoramidon (35 $\mu$M) was included (open circles) or excluded (closed circles) from the incubation mixture. An expanded view of the initial rates of modification is shown in the insert. The axis labels are the same for both figure and insert.

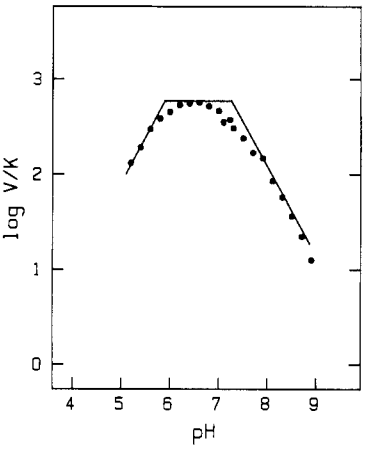


FIGURE 6: pH dependence of the hydrolysis of $1 \times 10^{-5}$ M dansyl-D-Ala-Gly-Phe($NO_2$)-Gly by NEP. Buffers used were MES (pH 5.2–7.2) and Tris-HCl (pH 7.1–9.1). Buffers were used at a concentration of 0.05 M and contained 0.2 M NaCl. $V/K$ is expressed in units of nmol $min^{-1}$ $mg^{-1}$. The lines drawn have slopes of 1, 0, and −1. Measurements were not made below pH 5 due to enzyme instability.

which both essential and nonessential residues are modified at the same rate can be determined by correlating the rate of loss of enzyme activity with the rate of modification of total reactive residues, eq 1. In this equation, $i$ is the number of

$$m = n[1 - (\%)^{1/i}] \quad (1)$$

essential residues, $n$ is the total number of residues modified over the time frame studied, $m$ is the number of residues modified at a given stage of modification, and (%) refers to the percent residual enzyme activity at a given stage of modification.

This equation has been used in a number of recent studies to determine the number of essential histidines modified by DEPC (Meyer & Cromartie, 1980; Dominici et al., 1985). In the present study, the rate of inactivation of NEP by DEPC was correlated with histidine modification in the same manner. As seen in Figure 4, a satisfactory fit to the linear portion of the data is obtained with $i = 1$ and $n = 2.8$. This indicates a rapid reaction of DEPC with three enzyme histidine residues of which only one is essential for activity. Also shown in Figure 4 are the theoretical curves for the presence of two ($i = 2$) or three ($i = 3$) essential histidine residues. Clearly, the data do not fit these situations. The deviation from linearity occurring in the lower portion of the graph could be attributed to deviation from pseudo-first-order kinetics due to reagent decomposition and/or to a slower reacting histidine becoming evident at these later time points. Similar observations have been made in several other enzyme systems in which a single essential histidine was modified by DEPC (Meyer & Cromartie, 1980; Saluja & McFadden, 1982; Cousineau & Meighen, 1976; Topham & Dalziel, 1986).

The above kinetic experiment was conducted over a time frame in which the enzyme was approximately 90% inactivated. Further evidence for a single essential histidine residue being modified at the active site was obtained by comparison of the full time course of DEPC modification monitored spectrally at 240 nm in the presence and absence of a 10-fold molar excess of the potent inhibitor phosphoramidon. As seen in Figure 5, approximately four histidines are modified by DEPC, with inclusion of phosphoramidon in the reaction mixture preventing the modification of one of these residues. The rate of histidine modification in the presence of phosphoramidon appeared slower than that in its absence throughout the time course of the reaction. This is illustrated in the insert in the figure, which shows an expanded view of the first 3 min of the time course of modification. The initial rates calculated from these points were 0.34 mol of His reacted per minute and 0.24 mol of His reacted per minute for the reactions without and with phosphoramidon, respectively. With three histidines reacting rapidly and one at a slightly slower rate, as suggested in the above paragraph, the decrease in the rate of histidine modification is consistent with the protection by phosphoramidon of one rapidly reacting residue.

*Effect of Modification on Kinetic Parameters.* The effect of DEPC modification on the kinetic properties of the enzyme was determined with enzyme partially inactivated by DEPC (10–20% residual activity). With glutaryl-Ala-Ala-Phe-4MeO2NA as substrate, an insignificant change in $K_m$ between the control (69 $\mu$M) and modified (52 $\mu$M) enzyme was observed. However, $V_{max}$ showed a large change between the control (3.5 $\mu$mol $min^{-1}$ $mg^{-1}$) and modified (0.54 $\mu$mol $min^{-1}$ $mg^{-1}$) enzyme. This result is consistent with complete inac-

tivation of the enzyme upon DEPC modification, with the residual activity representing unmodified enzyme.

*Dependence of Catalysis on pH.* Figure 6 shows the effect of varying pH on the kinetic parameter $V_{max}/K_m$. The slopes of the lines drawn were 1, 0, and −1, indicating two groups with approximate p$K_a$ values of 5.9 and 7.3. Since the substrate has no ionizing groups in the pH range studied, both of the ionizing groups can be attributed to the free enzyme form. Enzyme activity was not measured below pH 5 because of its instability in this pH region.

## Discussion

Although DEPC can react with the functional groups of a variety of amino acid residues, the usual group modified in neutral or slightly acidic media is the histidine imidazole. To provide evidence for an essential histidine in NEP, we have measured the absorbance change in the ultraviolet spectrum upon DEPC inactivation and found it to be characteristic of histidine modification, i.e., an increase in absorbance at 242 nm without a change at 280 nm (Miles, 1977). The second-order rate constant of DEPC inactivation is also similar to values obtained in previous studies of histidine modification with DEPC (see Results). In addition, it has been shown that mild treatment of the DEPC-modified enzyme with hydroxylamine completely and rapidly reverses inactivation, an observation that is considered strong evidence for histidine modification. Reaction of DEPC with lysine or arginine is irreversible, and tyrosine modification is considerably slower in its reversal (Miles, 1977). Reaction of a tyrosine residue can more definitively be excluded by the demonstration that acetylimidazole has no effect on enzyme activity. Cysteine and serine modification are not considered here since the absence of a reactive thiol or hydroxyl has been documented in previous studies (Kerr & Kenny, 1974b; Almenoff & Orlowski, 1983; Jackson & Hersh, 1986).

The pH dependence of inactivation was consistent with a single essential group with a p$K_a$ of 6.1. This is similar to values found for histidine in inactivation studies of other enzyme systems (Miles, 1977; Dominici et al., 1985) and is nearly identical with the lower kinetic p$K_a$ found when $V_{max}/K_m$ was plotted against pH (see below). The importance of this histidine in catalysis is indicated by a total loss of enzyme activity produced by DEPC modification. In addition, protection from DEPC inactivation by substrate with a $K_d$ similar to its $K_m$ points to the presence of this group at the active site of the enzyme. Although the possibility cannot be ruled out that modification of a histidine distal to the active site causes an inactive enzyme conformation, the results reported here are clearly consistent with an essential active site histidine in neutral endopeptidase 24.11.

As shown in this study, the pH dependence of $V_{max}/K_m$ reveals two essential ionizing groups with approximate p$K_a$ values of 5.9 and 7.3. These values are comparable to the kinetic p$K_a$ values of 5.9 and 7.5 found with thermolysin and other bacterial neutral proteases (Pangburn & Walsh, 1975), enzymes that are thought to catalyze cleavage of peptide bonds by a similar mechanism. In thermolysin, these amino acid residues have been identified as a glutamic acid (p$K_a$ = 5.9) and a histidine (p$K_a$ = 7.5). These residues in thermolysin have been proposed to act as acid/base catalysts, with glutamate a general base assisting water attack on the scissile bond and histidine a general acid protonating the amine leaving group (Pangburn & Walsh, 1975; Kunugi et al., 1982). More recently, however, crystallographic studies have suggested that the glutamic acid residue acts as a "proton shuttle" by promoting water attack and subsequently protonating the leaving nitrogen. The role of histidine in this revised mechanism is to stabilize the tetrahedral intermediate (Monzingo & Matthews, 1984).

In many bacterial and mammalian metallopeptidases previously studied, the proposed role of a general base or acid/base catalyst appears to be assumed by a glutamate residue. This is true in carboxypeptidases A and B (Vallee & Riordan, 1968; Hass & Neurath, 1971; Hass et al., 1972), thermolysin (Rasnick & Powers, 1978), other bacterial neutral proteases (Pangburn & Walsh, 1975), and angiotensin-converting enzyme (Harris & Wilson, 1982; Bunning et al., 1983). In these enzymes, the glutamic acid usually exhibits a kinetic p$K_a$ close to 6, a value probably indicating a hydrophobic environment surrounding the glutamic acid residue. In neutral endopeptidase 24.11, a likely candidate for this acid/base catalyst appears to be a histidine residue. This proposal is supported by the kinetic p$K_a$ of 5.9, which is nearly identical with kinetic p$K_a$ values in both thermolysin and carboxypeptidase A, and the evidence from DEPC inactivation indicating an essential role in catalysis by a histidine with a very similar p$K_a$. However, at this time the possibility that the histidine acts to stabilize the tetrahedral intermediate in the catalytic mechanism in a manner similar to that proposed for the histidine in thermolysin by Monzingo and Matthews (1984) cannot be ruled out.

The nature of the group exhibiting a kinetic p$K_a$ of 7.3 is not clear at this time. Cysteine, serine, and tyrosine seem unlikely candidates as previously discussed. More likely possibilities could be a zinc-bound water molecule or perhaps a glutamyl or aspartyl residue. There is also the possibility that this group reflects a second essential histidine. To investigate this possibility, the rate of histidine modification was correlated with enzyme inactivation at pH 7. As was observed at pH 5.8 (Figure 4), only a single essential histidine was modified. While this result disfavors a second essential histidine, it does not totally rule this out due to the possibility that this second histidine exhibits a low reactivity toward DEPC (Tsou, 1962) or cooperativity exists between residues (Rakitzis, 1978).

In summary, we have presented evidence for an essential histidine residue at the active site of neutral endopeptidase 24.11. The finding of this histidine presents an attractive target for future design of active site affinity labels and other mechanism-based inhibitors.

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# Identification of a Hydroxide Ligand at the Iron Center of Ribonucleotide Reductase by Resonance Raman Spectroscopy†

Britt-Marie Sjöberg,*,‡ Joann Sanders-Loehr,§ and Thomas M. Loehr§

*Department of Molecular Biology, Swedish University of Agricultural Sciences, Uppsala Biomedical Center, S-751 24 Uppsala, Sweden, and Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999*

*Received December 5, 1986; Revised Manuscript Received March 11, 1987*

ABSTRACT: The resonance Raman spectrum of protein B2 of ribonucleotide reductase from *Escherichia coli* shows several features related to its oxo-bridged binuclear iron center. A peak at 492 $cm^{-1}$ is assigned to the symmetric stretch of the Fe–O–Fe moiety on the basis of its 13-$cm^{-1}$ shift to lower energy upon $^{18}O$ substitution. The $^{18}O$ species shows an additional peak at 731 $cm^{-1}$, which is a good candidate for the asymmetric stretch of the Fe–O–Fe moiety. Its exact location in the $^{16}O$ species is obscured by the presence of a protein tryptophan vibration at 758 $cm^{-1}$. A third resonance-enhanced peak at 598 $cm^{-1}$ is identified as an Fe–OH vibration on the basis of its 24-$cm^{-1}$ shift to lower energy in $H_2{}^{18}O$, its 2-$cm^{-1}$ shift to lower energy in $D_2O$, and its pH-dependent intensity. A hydrogen-bonded $\mu$-oxo bridge similar to that in hemerythrin is suggested by the unusually low frequency for the Fe–O–Fe symmetric stretch and the 3-$cm^{-1}$ shift to higher energy of $\nu_s$(Fe–O–Fe) in $D_2O$. From the oxygen isotope dependence of $\nu_s$(Fe–O–Fe), an Fe–O–Fe angle of 138° can be calculated. This small angle suggests that the iron center consists of a tribridged core as in hemerythrin. A model for the binuclear iron center of ribonucleotide reductase is presented in which the hydroxide ligand sites provide an explanation for the half-of-sites reactivity of the enzyme.

Ribonucleotide reductase is the enzyme responsible for a balanced supply of precursors for DNA synthesis in all living organisms. One type of ribonucleotide reductase consists of two nonidentical subunits forming an enzymatically active one-to-one complex (Lammers & Follmann, 1983; Thelander & Reichard, 1979). The small subunit of this type of ribonucleotide reductase contains a unique prosthetic group, a tyrosyl radical stabilized by an adjacent binuclear iron center. In *Escherichia coli* the small subunit of ribonucleotide reductase, which contains two identical 43.5-kDa polypeptide chains, has been denoted protein B2. Over 10 years ago it was observed that the visible absorption spectrum of protein B2 was very similar to that of oxy and hydroxomet forms of the respiratory protein hemerythrin (Atkin et al., 1973; Garbett

† This work was supported by the Swedish Medical Research Council (B86-03X-06801), the Magnus Bergvall Foundation, and the National Institutes of Health (GM18865).
‡ Uppsala Biomedical Center.
§ Oregon Graduate Center.

0006-2960/87/0426-4242$01.50/0 