Gel Filtration Studies on the Binding of Peptides to Pepsin†

Esukapalli V. Raju, Robert E. Humphreys,‡ and Joseph S. Fruton*

ABSTRACT: Gel filtration studies on the binding of *p*-nitro-L-phenylalanyl-L-phenylalanine methyl ester [Phe(NO_2)-Phe-OMe] and of benzyloxycarbonyl-L-histidyl-L-phenylalanyl-L-phenylalaninol by pepsin and by chemically modified pepsin have been performed under conditions where ionic strength, buffer species, temperature, and pH have been varied. The results are consistent with the view that the interaction of the Phe(NO_2)-Phe unit of appropriate peptide substrates with the catalytic site of pepsin provides the principal binding energy in the enzyme–substrate interaction, and that this binding is largely hydrophobic in character.

In earlier work, a series of new synthetic substrates for pepsin was prepared (Inouye *et al.*, 1966; Inouye and Fruton, 1967). These compounds were of the type Z-His-X-Y-OMe (or OEt),[1] where X and Y are the amino acid residues linked by the pepsin-sensitive bond. This bond is cleaved most rapidly when X = L-phenylalanyl and Y = L-tryptophyl, L-phenylalanyl, or L-tyrosyl, the other member of the X-Y pair always being L-phenylalanyl (Trout and Fruton, 1969). The replacement of Phe in the X position of Z-His-Phe-Phe-OMe by Phe(NO_2) did not alter the kinetic parameters greatly, but the replacement of the terminal Phe-OMe unit by Pol made the X–Y bond resistant to pepsin action (Inouye and Fruton, 1967). Because Z-His-Phe(NO_2)-Pol has a high molar absorptivity and is resistant to pepsin action, this compound was selected as a substrate analog for equilibrium binding studies (Humphreys and Fruton, 1968); the gel filtration method refined in this laboratory (Fairclough and Fruton, 1966) was used. These studies showed the presence in pepsin of a primary binding site with high affinity for Z-His-Phe-(NO_2)-Pol, as well as secondary weaker binding sites. The proximity of the primary binding site to the catalytic site of pepsin was indicated by its abolition when pepsin was inactivated by the stoichiometric inhibitor tosyl-L-phenylalanyl-diazomethane (DPTB) (Delpierre and Fruton, 1966). On the other hand, treatment of pepsin with acetylimidazole, which enhances the rate of cleavage of Z-His-Phe-Phe-OEt (Hollands and Fruton, 1968) and reduces that of hemoglobin (Perlmann, 1966), did not abolish the primary binding site, but reduced the extent of binding of Z-His-Phe(NO_2)-Pol at the secondary sites.

The available knowledge regarding the side-chain specificity of pepsin has suggested that hydrophobic interactions play a predominant role in the formation of productive enzyme–substrate complexes (Tang, 1963; Trout and Fruton, 1969). In the present communication, data are reported on the effect of ionic strength, temperature, pH, and buffer species on the equilibrium binding of Z-His-Phe(NO_2)-Pol to pepsin; these data support the view that the interaction is largely hydrophobic in character. Furthermore, in view of earlier data (Inouye and Fruton, 1968) indicating that, in the interaction of a substrate such as Z-His-Phe-Phe-OMe with pepsin, the principal binding energy is derived from the interaction of the Phe-Phe-OMe unit with the catalytic site, the gel filtration method was applied to the study of the binding of the resistant dipeptide ester Phe(NO_2)-Phe-OMe by pepsin. Comparison of the data for this dipeptide ester to those obtained with Z-His-Phe(NO_2)-Pol supports the validity of the conclusions drawn from earlier work with the latter substrate analog.

Experimental Section

Materials. Crystalline swine pepsin (Worthington Biochemical Corp., lot PM 693-7) was purified in the manner described by Humphreys and Fruton (1968); when assayed with hemoglobin as the substrate (Delpierre and Fruton, 1965), the purified pepsin had a specific proteolytic activity of 2700 units/mg of protein. Acetyl-pepsin was prepared in the manner described previously (Humphreys and Fruton, 1968; Hollands and Fruton, 1968); this preparation contained an average of 4 acetyl groups per molecule of pepsin, and had a specific activity toward hemoglobin of 785 units/mg of protein. The DPTB-pepsin used in these studies was the preparation used in earlier work (Humphreys and Fruton, 1968). The concentration of protein was estimated spectrophotometrically at 278 nm. The molecular weight was assumed to be 35,000; the molar absorptivity of untreated pepsin and of DPTB-pepsin was 50,900, and that of acetyl-pepsin was 41,000.

The synthesis of Z-His-Phe(NO_2)-Pol (Humphreys and Fruton, 1968), and of Phe(NO_2)-Phe-OMe hydrobromide (Inouye and Fruton, 1967) has been described; the latter compound was kindly provided by Miss I. M. Voynick of this laboratory. The molar absorptivity at 278 nm and at pH 3–4 of these two compounds was found to be 9200 ± 100 (Cary 15 spectrophotometer).

Freshly prepared solutions of sodium formate, sodium acetate, or sodium butyrate buffer at pH 4.0 ± 0.05 or pH 3.0 ± 0.05 (Beckman expandomatic pH meter at room temperature) were used. The buffers were prepared by adding an equimolar solution of the acid to its sodium salt, and the ionic strength was adjusted by the addition of NaCl, when required.

† From the Kline Biology Tower, Yale University, New Haven, Connecticut 06520. *Received May 1, 1972.* These studies were aided by grants from the National Science Foundation (GB-18268) and from the National Institutes of Health (GM-18172).

‡ Medical Scientist Fellow of the Life Insurance Medical Research Fund.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445, 2485 (1966), are: Phe(NO_2), *p*-nitro-L-phenylalanyl; Pol, L-phenylalaninol; DPTB, L-1-diazo-4-phenyl-3-tosylamidobutanone (tosyl-L-phenylalanyl-diazomethane). Unless otherwise noted, the abbreviated designation of amino acid residues denotes the L form.

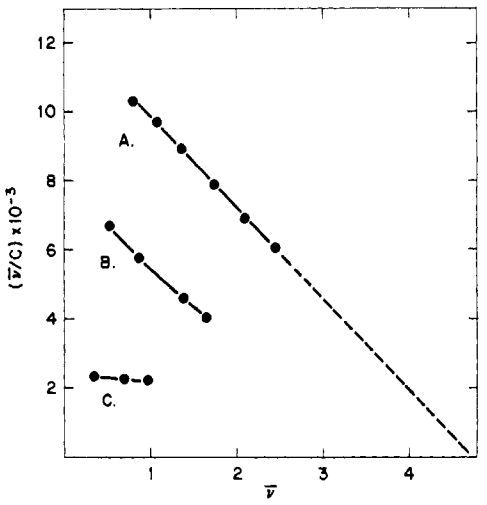

FIGURE 1: Scatchard plots for the binding of Phe(NO_2)-Phe-OMe by pepsin (curve A), acetyl-pepsin (curve B), and DPTB-pepsin (curve C) at pH 4.0 (formate buffer, $I = 0.2$) and 20°.

TABLE I: Binding of Phe(NO_2)-Phe-OMe to Pepsin.[a]

Phe(NO_2)-Phe-OMe (mM)	$\bar{\nu}$ Pepsin	$\bar{\nu}$ Acetyl-pepsin	$\bar{\nu}$ DPTB-pepsin
0.08	0.83	0.54	
0.11	1.07		
0.15	1.34	0.87	0.36
0.22	1.72		
0.30	2.07	1.41	0.70
0.40	2.45	1.67	0.93

[a] pH 4.0 (formate buffer, $I = 0.2$), 20°.

Methods. For the determination of the binding of Z-His-Phe(NO_2)-Pol and of Phe(NO_2)-Phe-OMe to pepsin, and to the chemically modified pepsins, the automated gel filtration procedure (Fairclough and Fruton, 1966) was employed. In the present studies, the dimensions of the Sephadex G-25 (medium) columns were 0.3 × 50 cm. The absorbance of the effluent solution at 278 nm was measured by means of a Gilford 2000 multiple absorbance recorder, equipped with Model 203 flow-cell assembly (0.2- and 0.5-cm cells), Model 208 auxiliary offset control, and Model 215 automatic blank compensator. The temperature was maintained within ±0.1° by circulating water around the column and cell compartment by means of a Forma-Temp Model 2095 constant-temperature bath. After the column had been equilibrated with the solution of peptide in buffer, as judged by a constant absorbance of the expected magnitude for the effluent solution, the protein (2 mg, corresponding to about 0.05 μmole, dissolved in 1 ml of the appropriate peptide–buffer solution) was added. The elution, at a flow rate of 3.0–3.5 ml/hr, was continued with the peptide–buffer solution until the absorbance had returned to its base-line value after the appearance of the trough in the elution pattern. With Z-His-Phe(NO_2)-Pol, the peptide concentration ranged between 0.08 and 1.0 mM. With Phe(NO_2)-Phe-OMe, the highest concentration that could be used was 0.4 mM; at pH 4.0 and 20°, higher concentrations of the dipeptide ester caused precipitation of the protein. Separate control experiments showed that at concentrations of 0.4 mM Phe(NO_2)-Phe-OMe, all the protein passed through Millipore filters and through the Sephadex column; at higher concentrations, significant amounts of protein were retained by the filter and by the column. Under the conditions of the present studies, the protein emerged at 2.4–2.5 ml in the elution pattern; for Phe(NO_2)-Phe-OMe the minimum of the trough was at 7.0 ml, and for Z-His-Phe(NO_2)-Pol it was 8.0 ml. From the area of the trough in the elution pattern and the flow rate, the amount of peptide bound in each experiment was determined, and the average number of moles of peptide bound per mole of protein ($\bar{\nu}$) was calculated in the manner described previously (Fairclough and Fruton, 1966). Nearly all runs were performed in triplicate, and the reported $\bar{\nu}$ values represent the mean of the three determinations; the deviation from the mean value usually was ±0.02. Separate control experiments showed that, under the conditions of these studies, there was no measurable cleavage of the peptides by pepsin during the time period (2–3 hr) required for the gel filtration runs.

Results

The data in Table I show that at pH 4.0, and at the highest concentration ($c = 0.4$ mM) of Phe(NO_2)-Phe-OMe that could be tested, pepsin can bind about 2.5 moles of the dipeptide ester/mole of pepsin, indicating multiple binding sites for this ligand. The plot of $\bar{\nu}/c$ *vs.* $\bar{\nu}$ (Scatchard, 1949) in Figure 1 suggests that the binding sites detected over the concentration range 0.08–0.4 mM are approximately equal in affinity. In view of the lack of binding data at higher concentrations of the ligand, linear extrapolation of this plot to the abscissa ($n = 4.8$) means only that there are at least five potential binding sites in pepsin for Phe(NO_2)-Phe-OMe. Linear extrapolation of the Scatchard plot to the ordinate gives a value of $nK = ca.\ 13 \times 10^3\ M^{-1}$ (K = association constant per binding site), but this estimate must be offered tentatively, because the presence of a single primary binding site of very high affinity may cause an upward curvature of the plot near the ordinate; this region corresponds to a concentration range of the dipeptide ester too low to give reliable binding data.

The extent of binding of Phe(NO_2)-Phe-OMe to acetyl-pepsin (Table I) is less than that foumd with unmodified pepsin, and the Scatchard plot suggests that, over the ligand concentration tested, approximately 1.6 fewer peptide molecules are bound per molecule of acetyl-pepsin (Figure 1). Since acetyl-pepsin has been shown to have enhanced catalytic activity (higher k_{cat}) and unchanged K_M toward synthetic substrates such as Z-His-Phe-Phe-OEt (Hollands and Fruton, 1968), it may be inferred that the decrease in the binding of Phe(NO_2)-Phe-OMe is a consequence of the blocking, by acetylation, of secondary binding sites. Whether the difference in binding results from the complete blocking of two sites, or from the partial inhibition of a larger number of sites, cannot be stated at present. In the case of DPTB-pepsin, where the catalytic site has been blocked by reaction with a single tosyl-L-phenylalanylmethyl group (Delpierre and Fruton, 1966), the extent of binding of Phe(NO_2)-Phe-OMe is greatly reduced (Table I), and the Scatchard plot appears to have an intercept on the ordinate of about $2.5 \times 10^3\ M^{-1}$.

Comparison of the extent of binding of Phe(NO_2)-Phe-OMe by the three pepsin preparations to the values previously obtained for Z-His-Phe(NO_2)-Pol at pH 4 and 20°

TABLE II: Effect of Buffer Species and Ionic Strength on Binding of Z-His-Phe(NO_2)-Pol by Pepsin.[a]

Buffer	RCOOH (M)	$RCOO^-$ (M)	NaCl (M)	I	$\bar{\nu}$
Formate	0.035	0.067	0.033	0.1	1.32
	0.074	0.133	0.067	0.2	1.55
	0.115	0.20	1.80	2.0	2.18
	1.15	2.00	0	2.0	1.95
Acetate	0.074	0.022	0.078	0.1	1.24
	0.147	0.044	0.156	0.2	1.39
Butyrate	0.094	0.021	0.079	0.1	0.80
	0.160	0.040	0.160	0.2	0.95

[a] Concentration of Z-His-Phe(NO_2)-Pol, 0.15 mM; pH 4.0; 20°.

TABLE III: Effect of Temperature and pH on the Binding of Z-His-Phe(NO_2)-Pol by Pepsin and Modified Pepsins.[a]

Protein	Temp (°C)	$\bar{\nu}$ pH 3.0	$\bar{\nu}$ pH 4.0
Pepsin	10	1.68	2.30
	20	1.44	1.65
	30	1.22	1.30
Acetyl-pepsin	10	0.87	1.33
	20	0.81	1.26
	30	0.58	1.11
DPTB-pepsin	10	0.08	0.66
	20	0.22	0.70
	30	0.09	0.57

[a] Concentration of Z-His-Phe(NO_2)-Pol, 0.20 mM; formate buffer (I = 0.2).

(Humphreys and Fruton, 1968) shows striking similarity in the magnitude of binding for comparable values of ligand and protein concentrations. This finding suggests that the removal of the Z-His group and the replacement of the carbinol group by a carboxymethyl group do not alter greatly the affinity of the ligand to pepsin, and supports the view that the principal contribution to binding energy is provided by the interaction of the Phe(NO_2)-Phe unit with the protein.

That this interaction is predominantly hydrophobic in character is indicated by the data for the effect of buffer species and ionic strength on the binding of Z-His-Phe(NO_2)-Pol. It will be noted in Table II that at pH 4 (formate buffer), a 10-fold increase in ionic strength (from 0.2 to 2.0) causes a significant increase in the extent of binding. Furthermore, at a given ionic strength, there is progressively less binding of the ligand when the buffer species is changed from formate to acetate to butyrate. It is noteworthy that the largest change is observed upon replacing acetate by butyrate, whose acids have nearly the same pK_a value, whereas less difference is found between formate buffer and acetate buffer, despite the large difference in the pK_a values of formic acid (3.9) and of acetic acid (4.7). It may be concluded, therefore, that in the concentration range of buffer used, the inhibitory effect of acetate and butyrate is not primarily related to their anionic character but to the interaction of their alkyl groups with pepsin. This inhibition of binding may be considered to be comparable to the inhibition of pepsin action on small synthetic substrates by aliphatic alcohols (Tang, 1965; Zeffren and Kaiser, 1967), aromatic alcohols and acids (Schlamowitz *et al.*, 1968), and other organic solvents (Cornish-Bowden and Knowles, 1969).

The data in Table III show that in the interaction of Z-His-Phe(NO_2)-Pol with active pepsin the extent of binding is markedly decreased as the temperature is raised from 10 to 30°, but that the binding of the peptide to the modified pepsin is less temperature dependent. It will also be noted from Table III that the binding of Z-His-Phe(NO_2)-Pol to pepsin is less at pH 3 than at pH 4. This finding is consistent with the observation that, in the cleavage of cationic substrates by pepsin, the kinetically determined value of K_M (assumed to approximate the dissociation constant of the enzyme–substrate complex) is usually lower near pH 4 than near pH 3 (Hollands *et al.*, 1969; Sachdev and Fruton, 1969). In view of the large number of carboxyl groups in pepsin (Rajagopalan *et al.*, 1966), it may be surmised that the increasing binding of cationic peptide derivatives at pH 4 may be a consequence of the contribution of enzymic carboxylate groups to electrostatic interaction with the cationic group brought into the complex by the strong hydrophobic interaction with the Phe(NO_2)-Phe unit. The data in Table III refer to a ligand concentration of 0.20 mM; similar effects of temperature and pH were noted at concentrations of Z-His-Phe(NO_2)-Pol of 0.08 and 0.35 mM.

Discussion

Upon the conversion of pepsinogen to pepsin, the protein acquires the ability to bind a substrate analog at several sites, the affinities of which may be modified in chemical derivatives. The affinity at the primary binding site for Z-His-Phe(NO_2)-Pol may be reduced greatly by the stoichiometric inhibitor DPTB which reacts at the catalytic site of pepsin; because the binding pattern of Phe(NO_2)-Phe-OMe closely resembles that of Z-His-Phe(NO_2)-Pol, it may be inferred that the binding energy at the principal binding site, or catalytic site of pepsin, is derived from the interaction of the two apolar aromatic side chains with complementary regions of the enzyme. This finding is consistent with previous kinetic studies (Inouye *et al.*, 1966; Inouye and Fruton, 1968; Trout and Fruton, 1969) that two adjacent apolar side chains in either a substrate or inhibitor are required for strong binding of a peptide at the catalytic site of pepsin.

Although the data are consistent with the view that hydrophobic interactions play a major role in the binding of the side chains of the Phe(NO_2)-Phe unit at the primary catalytic site of pepsin, the presence of additional binding sites makes it difficult to interpret unambiguously the effects of ionic strength, buffer species, temperature, and pH. If a secondary binding site is adjacent to the catalytic site, the competition for occupancy of the two sites will make the interaction more complex than can be accounted for in terms of the presence of multiple independent binding sites. Moreover, the possibility exists that changes in environmental factors may perturb the structure of the protein in ways that will affect the binding at different sites in a nonuniform manner. More specific probes for pepsin–peptide interaction are therefore needed; one approach is the use of fluorescent probes as in recent studies in this laboratory (Sachdev *et al.*, 1972).

References

Cornish-Bowden, A. J., and Knowles, J. R. (1969), *Biochem. J. 113*, 353.
Delpierre, G. R., and Fruton, J. S. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 1161.
Delpierre, G. R., and Fruton, J. S. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1817.
Fairclough, G. F., and Fruton, J. S. (1966), *Biochemistry 5*, 673.
Hollands, T. R., and Fruton, J. S. (1968), *Biochemistry 7*, 2045.
Hollands, T. R., Voynick, I. M., and Fruton, J. S. (1969), *Biochemistry 8*, 575.
Humphreys, R. E., and Fruton, J. S. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 519.
Inouye, K., and Fruton, J. S. (1967), *Biochemistry 6*, 1765.
Inouye, K., and Fruton, J. S. (1968), *Biochemistry 7*, 1611.
Inouye, K., Voynick, I. M., Delpierre, G. R., and Fruton, J. S. (1966), *Biochemistry 5*, 2473.
Perlmann, G. (1966), *J. Biol. Chem. 241*, 153.
Rajagopalan, T. G., Moore, S., and Stein, W. H. (1966), *J. Biol. Chem. 241*, 4940.
Sachdev, G. P., and Fruton, J. S. (1969), *Biochemistry 8*, 4231.
Sachdev, G. P., Johnston, M. A., and Fruton, J. S. (1972), *Biochemistry 11*, 1080.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schlamowitz, M., Shaw, A., and Jackson, W. T. (1968), *J. Biol. Chem. 243*, 2821.
Tang, J. (1963), *Nature (London) 199*, 1094.
Tang, J. (1965), *J. Biol. Chem. 240*, 3810.
Trout, G. E., and Fruton, J. S. (1969), *Biochemistry 8*, 4183.
Zeffren, E., and Kaiser, E. T. (1967), *J. Amer. Chem. Soc. 89*, 4204.

Nuclear Magnetic Resonance Studies of Carbonic Anhydrase. Binding of Sulfacetamide to the Manganese Enzyme†

Amos Lanir and Gil Navon*

ABSTRACT: The effect of manganese(II)–bovine carbonic anhydrase on the proton magnetic relaxation times T_1 and T_2 of sulfacetamide was studied. The activity and stability of the manganese–enzyme complex were determined. The specificity of sulfacetamide binding to the active site was demonstrated by its replacement by *p*-toluenesulfonamide and azide ion. The equilibrium constant for the inhibitor–enzyme complex determined by the nuclear magnetic resonance method was found to agree with that obtained by kinetic measurements, providing further evidence for the specificity of the binding. The enhancement of the relaxation rate, $1/T_2$, appeared to be controlled by both the exchange lifetime and the dipolar relaxation mechanisms. Their relative contributions were separated using the measured T_1/T_2 ratios and the correlation time for the dipolar interaction could be determined. It was found that the electronic spin relaxation is the dominating correlation time at lower temperatures, while the rotational correlation time, which has a different temperature dependence, dominates at higher temperatures. Substituting the correlation times in the Solomon–Bloembergen equations for relaxation rates of the bound inhibitor, the distances between the manganese ion and protons of the methyl group, and phenyl protons ortho and meta to the sulfonamide group in the bound inhibitor molecule were calculated as 4.6 ± 0.2, 5.6 ± 0.3, and 6.6 ± 0.4 Å, respectively. These distances fit with a model in which the sulfonamide nitrogen is directly bound to the metal ion. However, this model is not unique. From the exchange lifetime and the equilibrium binding constants, assuming one-step inhibition mechanism, the rate constants for the association and dissociation reactions and their temperature dependences were derived.

Sulfonamides are known to be potent inhibitors of the zinc metalloenzyme, carbonic anhydrase (EC 4.2.1.1) (Maren, 1967). X-Ray crystallography has shown that sulfonamide inhibitors are bound in a cavity in the enzyme close to the zinc atom (Fridborg *et al.*, 1967; Liljas *et al.*, 1969). Still the detailed structure of the enzyme–inhibitor complex is not yet available. There are several other spectroscopic indications that sulfonamides are bound near the metal and probably occupy a position within the coordination sphere (Lindskog, 1963; Lindskog and Nyman, 1964; Coleman, 1967b, 1968). In the complex the sulfonamides are in the anionic state, although there are still different arguments on whether the deprotonation step occurs before or after the binding process (Kernohan, 1966; Chen and Kernohan, 1967; Lindskog and Thorslund, 1968; Lindskog, 1969; King and Burgen, 1970; Taylor *et al.*, 1970b). Fluorescence shifts observed with dansylamide (Chen and Kernohan, 1967) and nuclear magnetic resonance (nmr) studies of sulfonamide binding (Lanir and Navon, 1971; Navon and Lanir, 1972) confirmed the stabilization of the complex through hydrophobic forces involving the aromatic ring which is tightly bound to the enzyme. In order to be highly effective inhibitors, the sulfonamides must possess an unsubstituted sulfonamide group (Maren, 1967), although certain modifications of this group can be made without destroying the binding affinity completely (Krebs, 1948; Whitney *et al.*, 1967; Pocker and Stone, 1968).

The zinc in the native carbonic anhydrase can be reversibly replaced by various bivalent metal ions of the first transition series (Lindskog and Malmstrom, 1962; Lindskog, 1963).

† From the Department of Chemistry, Tel-Aviv University, Tel-Aviv, Israel. *Received April 27, 1972.*