Inhibition of Lysozyme by Some Copolymers of Amino Acids*

MICHAEL SELA AND LISA A. STEINER†

From the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel

Received October 25, 1962

Copolymers of glutamic acid and tyrosine, phenylalanine or leucine, are powerful inhibitors of the enzymatic action of lysozyme. The homopolymer, polyglutamic acid, is less effective as an inhibitor than the copolymers. The inhibition, which is especially effective at *p*H values lower than 6 and at low salt concentrations, may be reversed by polylysine and appears to depend in part on electrostatic interaction between the basic enzyme and the acidic copolymer. The increased efficiency of inhibition obtained by the inclusion within the inhibitor of certain uncharged amino acids indicates that other, nonionic bonds are also involved in the interaction. The inhibition is reversed in concentrated urea solutions.

Inhibition of enzymes plays an important role in the regulatory mechanisms of cell metabolism. Natural macromolecular enzyme inhibitors have been described (Herriott, 1941; Laskowski and Laskowski, 1954), and for the action of some of them an initial polyelectrolytic interaction of oppositely charged enzyme and inhibitor molecules has been postulated (Green and Work, 1953). Studies on the inhibition of basic enzyme molecules with polyanions and of acidic enzyme molecules with polycations have been reported (for literature see, *e.g.*, Coleman and Edelhoch, 1956; Mora and Young, 1959; Sela and Katchalski, 1959). Polyelectrolytic synthetic polypeptides have served on several occasions as model compounds in these investigations (Katchalski *et al.*, 1954; Vandendriessche, 1956; Sela and Katchalski, 1959; Kornguth and Stahmann, 1960; Sela, 1962).

The purpose of the present study was to elucidate the influence on the inhibitory efficiency of the inclusion within a polyanionic inhibitor of some amino acids with no charged groups in their side-chains. It was reported previously that copolymers of glutamic acid and the aromatic amino acids, tyrosine or phenylalanine, are much more efficient inhibitors of ribonuclease than polyaspartic acid (Sela, 1962). It was assumed that the greater inhibitory efficiency, as compared to the polymer composed exclusively of negatively charged residues, is due to an interaction of the aromatic amino acids with specific groups in the ribonuclease molecule over and above the long-range electrostatic interaction between the inhibitor and the enzyme which is responsible for the inhibitory capacity of polyanions in general. The specific short-range bonds were disrupted by 8 M urea, in which the copolymers were not better inhibitors than the macroanions lacking the aromatic side-chains. The addition of a copolymer of L-glutamic acid and L-tyrosine in a residue molar ratio of 1:1 not only prevented the digestion by ribonuclease of *E. coli* RNA of high molecular weight, but also stopped it at any moment desired (Littauer and Sela, 1962).

Some of the six tyrosine residues in ribonuclease are known to exist in a form which contributes in an anomalous way to the ultraviolet spectrum (Sela *et al.*, 1957) and makes them unavailable for spectrophotometric titration (Shugar, 1952; Tanford *et al.*, 1955). It has been assumed, therefore, that these residues are of importance in keeping the molecule in its active conformation. Thus, it seems possible that the presence of the aromatic side-chains in the polyanionic molecule resulted in an increased inhibitory efficiency in the case of ribonuclease because of specific interference with bonds involving some tyrosine residues of the enzyme. In order to find out whether the additional efficiency of inhibition due to the presence of aromatic amino acids is specific for ribonuclease or whether it is a more general phenomenon characteristic of the inhibition of basic proteins with biological activity by polyacids, we have investigated now the inhibition of lysozyme by copolymers of glutamic acid and tyrosine or phenylalanine. A copolymer of glutamic acid and leucine was also checked in order to determine whether the additional efficiency is the result of a specific interaction involving aromatic side-chains, or some other process, *e.g.*, hydrophobic bonding.

Lysozyme is inhibited by the acidic polymers hyaluronic acid, pneumococcus polysaccharide, RNA, DNA, natural poly-γ-glutamic acid (Skarnes and Watson, 1955), and heparin (Kaiser, 1953), while the basic macromolecules histone, protamine (Kaiser, 1953), and polylysine (Shalitin, 1959) activate the enzyme, possibly because of their interaction with the negatively charged bacterial cell wall (Katchalski *et al.*, 1953).

Results described below indicate that copolymers of glutamic acid and of tyrosine, phenylalanine, or leucine are indeed under a variety of conditions much more effective inhibitors of lysozyme than the homopolymer of glutamic acid. The increase in the inhibitory efficiency is thus of a more general character both as far as the nature of the enzyme and as far as the nature of the side-chains of the amino acid residues included in the inhibitor are concerned.

Materials and Methods

Materials.—Egg white lysozyme, recrystallized twice, was a gift from Dr. W. F. Harrington. Bacto-Lysozyme Substrate, a standardized ultraviolet-killed dried culture of *Micrococcus lysodeikticus* prepared according to Smolelis and Hartsell (1949), was obtained from Difco Laboratories, Detroit, Mich.

Poly-L-glutamic acid (average degree of polymerization, *n* 600) and poly-L-lysine hydrobromide (*n* 115) were obtained from the departmental collection. Copolymers are denoted by the letter *p* followed by brackets including the component L-amino acids and the residue molar ratio. *p*(Tyr,Glu; 1:1), *n* 85, was described by Sela *et al.* (1962). *p*(Tyr,Glu; 1:4), *n* 31; *p*(Tyr,Glu; 1:9), *n* 88; and *p*(Phe,Glu; 1:0.87), *n* 142, were described by Sela and Arnon (1960). *p*(Tyr,Lys; 1:1), *n* 1250, was a gift from Dr. M. Rigbi. *p*(Glu,Leu; 1:1.2), *n* 190, was prepared in analogy with the synthesis of linear copolymers given by Sela *et al.* (1962). The number average degrees of polymerization were obtained from amino-nitrogen (Van Slyke) determinations. The copolymer with lysine was checked before the removal of the carbobenzoxy functions protecting the ε-amino groups.

* This work was supported by research grants (A-3083 and 235103) from the National Institutes of Health, United States Public Health Service.

† Fellow of the Helen Hay Whitney Foundation.

All the experiments were carried out either in sodium phosphate or in Tris-malonate buffers. In the latter case the ionic strength was adjusted with sodium chloride, when necessary.

Lysozyme Assay.—Lysozyme was assayed by a modification of the method of Smolelis and Hartsell (1949), based on the decrease in turbidity of a suspension of bacterial cells following the addition of lysozyme. Bacto-Lysozyme Substrate was suspended in buffer, so that the absorbancy of the suspension as measured in a Beckman model DU spectrophotometer at 540 mμ was approximately 0.8. Usually, 50 mg of substrate was added to 100 ml of buffer, the large particles were allowed to settle out, and the remainder was mixed thoroughly. A portion of the substrate suspension was added to an equal volume of the test solution containing lysozyme or lysozyme and inhibitor. The absorbancy at 540 mμ was measured immediately after mixing and then at short intervals for 5 minutes. Enzymatic activity was determined by comparing the changes in absorbancy with time of the unknown solution and of a series of lysozyme standards.

Results

The inhibition of lysozyme by several anionic polyamino acids is illustrated in Figure 1. None of the polyanions investigated caused any decrease in turbidity of the substrate when mixed with it in the absence of enzyme. Poly-L-glutamic acid in 0.1 M phosphate buffer of *p*H 6.2 caused only a partial inhibition of the enzyme even at an inhibitor-to-enzyme ratio (*I*:*E*) (weight per weight) of 100. On the other hand *p*(Tyr,Glu; 1:1) was a very good inhibitor under the same conditions, causing 50% inhibition at *I*:*E* = 0.5. The same result was obtained for *p*(Phe,Glu; 1:0.87), while *p*(Glu,Leu; 1:1.2) was only slightly less efficient as an inhibitor of lysozyme (50% inhibition at *I*:*E* = 1). Copolymers of tyrosine and glutamic acid containing relatively few tyrosine residues, *p*(Tyr,Glu; 1:4) and *p*(Tyr,Glu; 1:9), were not better inhibitors than the homopolymer of glutamic acid (Fig. 1).

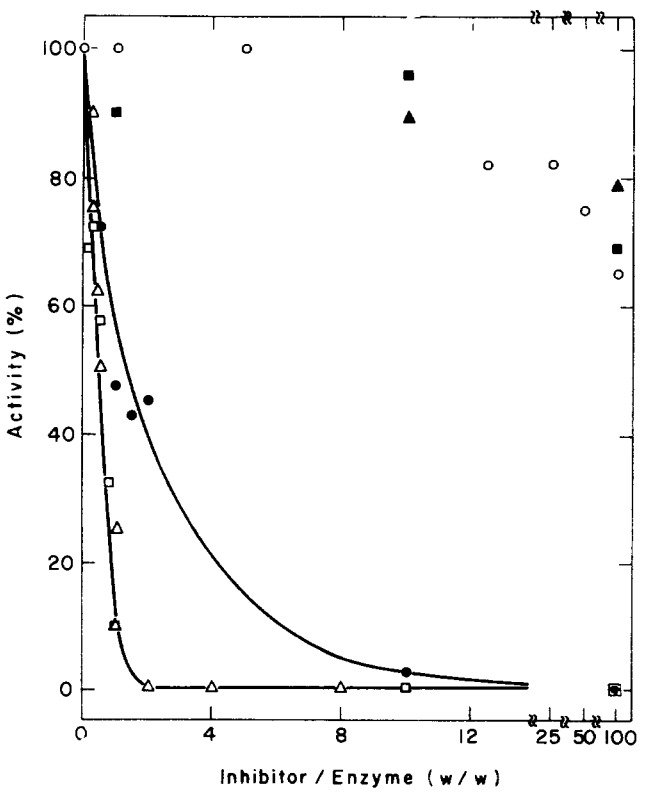

FIG. 1.—Decrease in the enzymatic activity of lysozyme as a function of inhibitor added. The assays were carried out in 0.1 M sodium phosphate buffer at *p*H 6.2. The concentration of the enzyme was 4 μg/ml. O, poly-L-glutamic acid; △, *p*(Tyr,Glu; 1:1); ▲, *p*(Tyr,Glu; 1:4); ■, *p*(Tyr,Glu; 1:9); □, *p*(Phe,Glu; 1:0.87); ●, *p*(Glu,Leu; 1:1.2).

A mixture of the free amino acids tyrosine and glutamic acid had no inhibitory effect on lysozyme even when each of the two amino acids was present in a 25-fold weight excess over the enzyme. A mixture of free tyrosine and polyglutamic acid, each again present in 25-fold excess, was not a better inhibitor than polyglutamic acid alone.

p(Tyr,Glu; 1:1), at *I*:*E* = 10, when added 2 minutes after the start of the reaction to a solution containing both the substrate and the enzyme in Tris-malonate buffer of *p*H 6.0 and ionic strength 0.1, inhibited the reaction completely. This effect of stopping the enzymatic reaction was not obtained at *I*:*E* = 1, even though at this ratio the addition of the inhibitor to the enzyme before the substrate caused complete enzymatic inhibition.

Dependence of Inhibition on pH.—The dependence of the activity of lysozyme on *p*H was investigated by Dickman and Proctor (1952), who found the optimal activity to occur in the *p*H range 5.0 to 7.0. In similar experiments we observed a broad *p*H optimum of activity between *p*H 5.5 and 8.0. Significant activity was observed at *p*H values as low as 4.5 and as high as 9.0.

The inhibition of lysozyme activity by polyglutamic acid and *p*(Tyr,Glu; 1:1) was investigated in the *p*H range 4.5–9.0. As illustrated in Figure 2, polyglutamic acid, at *I*:*E* = 10, completely lost its inhibitory capacity above *p*H 6.0, while *p*(Tyr,Glu; 1:1) exhibited full inhibition at this inhibitor-to-enzyme ratio at all the *p*H values investigated. At *I*:*E* = 1, *p*(Tyr,Glu; 1:1) inhibited completely the enzymatic activity below *p*H 6 and depressed lysozyme activity even at *p*H 9.

Dependence of Inhibition on Ionic Strength.—The activity of lysozyme as a function of ionic strength is given in Figure 3. Measurements were carried out in Tris-malonate buffer of *p*H 6.0, adjusted with sodium chloride to the desired ionic strength.

The ionic strength dependence of the inhibition of lysozyme by polyglutamic acid and by *p*(Tyr,Glu; 1:1) is shown in Figure 4. *p*(Tyr,Glu; 1:1) inhibited completely at *I*:*E* = 1 even at the highest ionic strength investigated, whereas at *I*:*E* = 0.5 the inhibition was strongly dependent on ionic strength in the region up to 0.1. Above this ionic strength the degree of inhibition was no longer influenced by the salt content of the solution. At *I*:*E* = 1, polyglutamic acid inhibited lysozyme only when the ionic strength was less than 0.1.

Effect of Polylysine.—From data summarized in Table I it is seen that the addition of small amounts of poly-L-lysine hydrobromide enhanced the enzymatic activity of lysozyme, as reported by Shalitin (1959). At higher polybase concentrations, partial inhibition of the catalytic activity occurred. At the highest polylysine concentration used, agglutination of the killed bacterial cells which served as substrate was apparent, in agreement with the findings of Katchalski *et al.* (1953) on the agglutination of *E. coli* and *M. pyogenes* by polylysine.

The inhibition of lysozyme by the polyanions investigated may be reversed by polylysine. The effect of polylysine on the inhibition of lysozyme by *p*(Tyr,Glu; 1:1) at *p*H 6.0 is shown in Table I. The addition

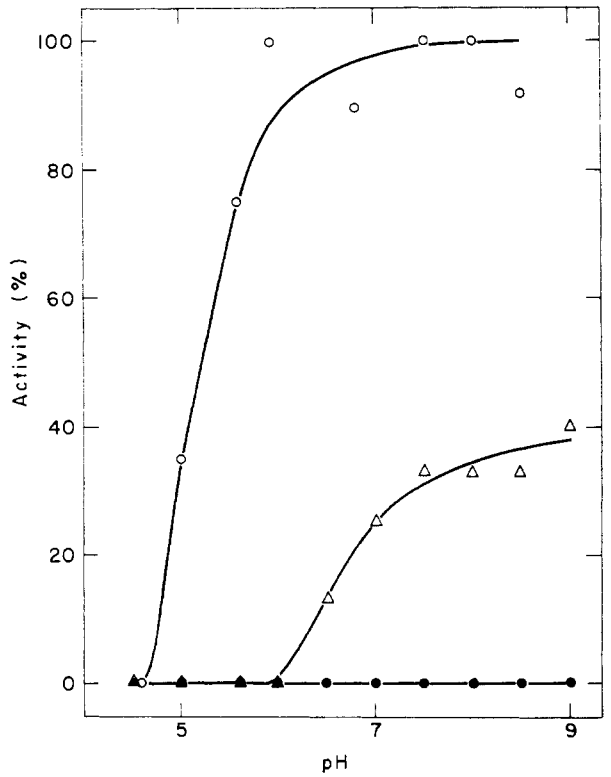

FIG. 2.—Enzymatic activity of lysozyme as a function of *p*H in presence of: O, polyglutamic acid at $I:E$ = 10; △, *p*(Tyr,Glu; 1:1) at $I:E$ = 1; ●, *p*(Tyr,Glu; 1:1) at $I:E$ = 10. The assays were carried out in 0.025 M Tris and 0.025 M malonic acid, adjusted with sodium hydroxide to the desired *p*H and with sodium chloride to an ionic strength of 0.1. The concentration of the enzyme at *p*H 6.0 was 4 μg/ml, while at the other *p*H values the amount of enzyme was adjusted so as to give an absolute activity similar to that obtained at *p*H 6.0. The activity in the absence of inhibitor is considered at each *p*H value as 100%.

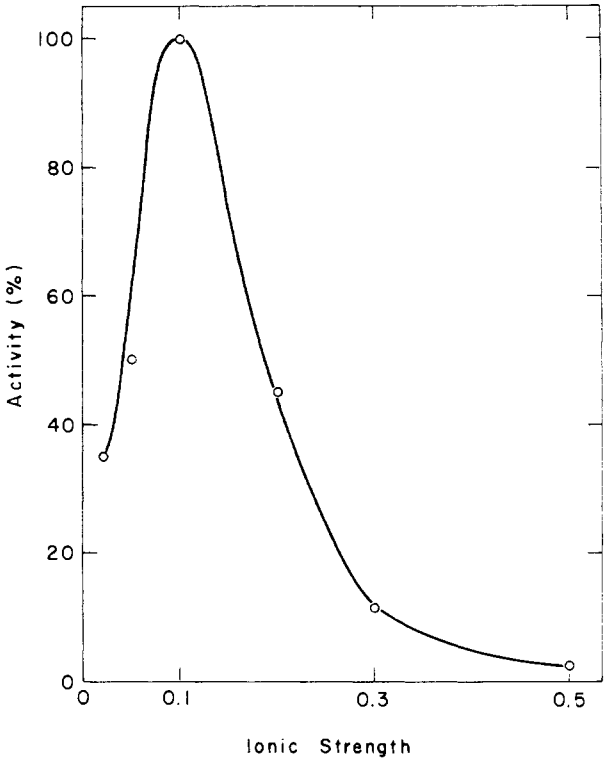

FIG. 3.—Enzymatic activity of lysozyme as a function of ionic strength. The assays were carried out in 0.0075 M Tris and 0.0075 M malonic acid, adjusted with sodium hydroxide to *p*H 6.0 and with sodium chloride to the ionic strength indicated. The concentration of the enzyme at an ionic strength of 0.1 was 4 μg/ml, while at the other ionic strengths the amount of enzyme was adjusted so as to give an absolute activity similar to that obtained at an ionic strength of 0.1.

TABLE I

EFFECT OF POLY-L-LYSINE ON THE ENZYMATIC ACTIVITY OF LYSOZYME IN THE ABSENCE OR PRESENCE OF *p*(Tyr,Glu; 1:1)[a]

p(Tyr,Glu; 1:1) (μg/ml)	Polylysine Hydrobromide (μg/ml)	Activity (%)
0	0	100
0	1	150
0	2	150
0	6	125
0	10	50
0	16	25
0	25	0
0	50	0
1	0	50
2	0	0
2	0.5	0
2	1	75
2	2	145
2	5	110
2	10	60
2	16	25
6	6	175
10	2.5	0
10	5	80
10	10	90
10	15	50
10	25	0

[a] The experiments were carried out in Tris-malonate buffer of *p*H 6.0 and ionic strength 0.1. The concentration of lysozyme was 2 μg/ml throughout.

of poly-L-lysine hydrobromide in an amount equal in weight to that of the inhibitor caused complete reversal of inhibition. The inhibitory effect of *p*(Glu,Leu; 1:1.2) at $I:E$ = 1 was similarly reversed by an amount of polylysine equal in weight to that of the inhibitor. The inhibition of lysozyme at *p*H 4.5 brought about either by polyglutamic acid or by *p*(Tyr,Glu; 1:1) was also reversed by polylysine at the same weight ratio.

Polylysine may react both with the polyanionic inhibitor and the negatively charged cell surface. This was seen when, under the conditions described in Table I, 10 μg of polylysine hydrobromide was added, either in the absence of the polyanion, causing a decrease of activity to 50% of that of the standard, or in the presence of 10 μg of the polyanion, when the activity was 90%.

A copolymer containing both tyrosine and lysine was investigated in order to determine whether its action was similar to that of polylysine or whether the presence of tyrosine would convert it into an inhibitor. The results are summarized in Table II. *p*(Tyr,Lys; 1:1) neither inhibited nor activated the enzyme except for the inhibition observed when it was used in large excess. The reversal by *p*(Tyr,Lys; 1:1) of the inhibition of lysozyme by *p*(Tyr,Glu; 1:1) was significantly less efficient than that obtained with polylysine.

Free lysine had no effect whatsoever either on the activity of lysozyme or on the inhibitory effect of *p*(Tyr,Glu; 1:1) when checked at weight ratios of lysine to enzyme or inhibitor of 1 and 8.

Inhibition in Aqueous Urea.—Lysozyme is catalytically active in urea solutions (Leonis, 1956), but in our experience the enzyme must be added to the killed

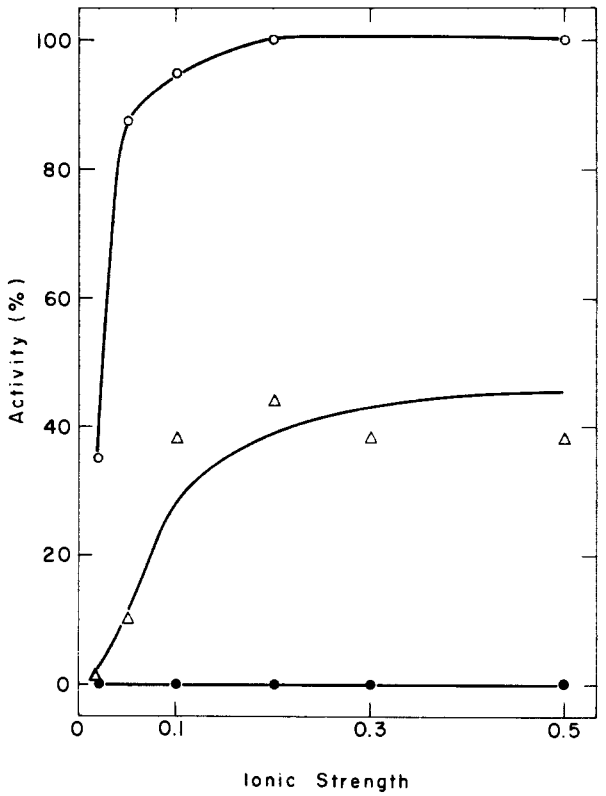

FIG. 4.—Enzymatic activity of lysozyme as a function of ionic strength in presence of: O, polyglutamic acid at $I:E$ = 1, △, p(Tyr,Glu; 1:1) at $I:E$ = 0.5; ●, p(Tyr,Glu; 1:1) at $I:E$ = 1. The assays were carried out in 0.0075 M Tris and 0.0075 M malonic acid, adjusted with sodium hydroxide to pH 6.0 and with sodium chloride to the ionic strength indicated. Amounts of enzyme were as in Fig. 3. The activity in the absence of inhibitor is considered, at each ionic strength, as 100%.

TABLE II

EFFECT OF p(Tyr,Lys; 1:1) ON THE ENZYMATIC ACTIVITY OF LYSOZYME IN THE ABSENCE OR PRESENCE OF p(Tyr,Glu; 1:1)[a]

p(Tyr,Glu; 1:1) (μg/ml)	p(Tyr,Lys; 1:1) (μg/ml)	Activity (%)
0	0	100
0	0.25	100
0	1	105
0	2	105
0	4	112
0	20	75
0	200	15
4	0	0
4	4	10
4	50	65

[a] The experiments were carried out in Tris-malonate buffer of pH 6.0 and ionic strength 0.1. The concentration of lysozyme was 4 μg/ml throughout.

bacterial cells almost immediately after their suspension in aqueous urea, as otherwise no significant activity can be detected. The addition of urea to the substrate causes an initial decrease in turbidity which must be taken into account in comparing activity data with those obtained in the absence of urea.

The activity of lysozyme in the presence of p(Tyr,-Glu; 1:1) at $I:E$ = 1 was measured in aqueous urea solutions. As seen in Figure 5 an increase in the concentration of urea at pH 6.0 caused a dramatic decrease in the efficiency of inhibition, and in 6 M urea lysozyme was as active in the presence as in the absence of

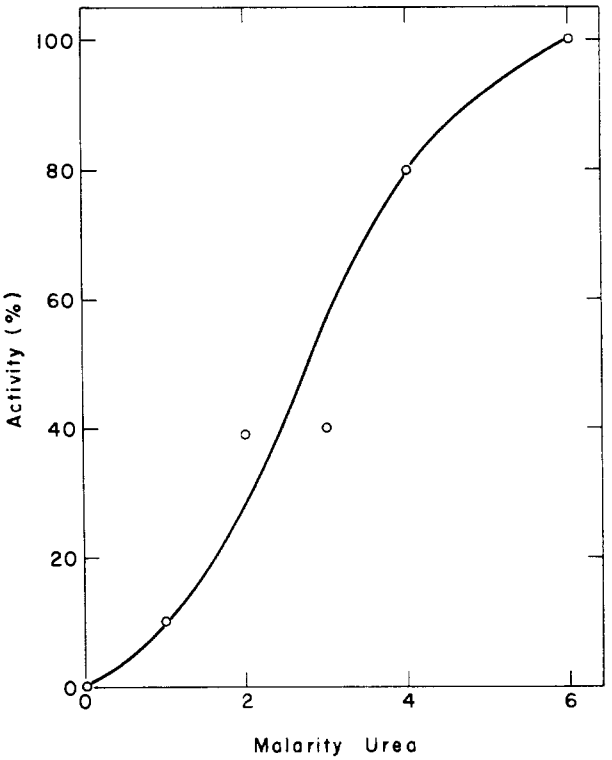

FIG. 5.—Enzymatic activity of lysozyme as a function of concentration of urea in presence of p(Tyr,Glu; 1:1) at $I:E$ = 1. The assays were carried out in 0.025 M Tris and 0.025 malonic acid, adjusted with sodium hydroxide to pH 6.0 and with sodium chloride to an ionic strength of 0.1. The concentration of the enzyme was 4 μg/ml. The activity in the absence of inhibitor is considered at each concentration of urea as 100%.

p(Tyr,Glu; 1:1). A similar decrease was observed in urea when the inhibitor was p(Glu,Leu; 1:1.2) at $I:E$ = 1, pH 6.0, and ionic strength 0.1. The activity increased in this case from 35% in the absence of urea to 85% in 4 M urea.

Polyglutamic acid could not be compared with p(Tyr,Glu; 1:1) under the above conditions, as at pH 6.0 it has no inhibitory effect. Experiments carried out at pH 5.3 (Tris-malonate buffer, ionic strength of 0.1) showed that in 6 M urea there was an increase in the activity of the system containing p(Tyr,Glu; 1:1) at $I:E$ = 1 from 0% to 70%, while the activity of the system containing polyglutamic acid at $I:E$ = 10 increased from 50% to 75%.

Inhibitor-Enzyme Complexes.—The inhibitory effect of the polymers described results from their interaction with the enzyme. In the range of concentrations used in this study, mixing of inhibitor and enzyme yielded solutions that were completely clear. In order to check the possibility that aggregates were formed as the result of the interaction of enzyme and inhibitor, even though no turbidity was observed, the mixtures were investigated in the following manner. The solution containing lysozyme and inhibitor was run in a Servall refrigerated centrifuge at 8000 rpm for 45 minutes. The upper half of the centrifuged solution was then separated from the lower half, polylysine was added to each fraction, and the assay was performed as usual. Thus, whenever an inactive inhibitor-enzyme complex was present, polylysine would reactivate the enzyme.

When a solution containing p(Tyr,Glu; 1:1) and lysozyme at pH 6.0 and ionic strength 0.1 was treated in this manner, all the activity, after reactivation with polylysine, was found in the lower half of the solution.

Similarly, when polyglutamic acid was mixed with lysozyme at *p*H 4.5 (Tris-malonate buffer, ionic strength of 0.1) and centrifuged, all the activity was recovered in the lower half. When an experiment of the same type was performed in 6 M urea with *p*(Tyr,Glu; 1:1) at *I*:*E* = 1 (*p*H 6.0 and ionic strength of 0.1), conditions under which the polyanion exhibits no inhibitory effect (Fig. 5), the same amount of activity was recovered in the upper as in the lower half of the centrifuged solution. Thus, it seems that the inhibition of lysozyme by the polyanions investigated is accompanied by the formation of enzyme-inhibitor complexes large enough to be sedimented at 8000 rpm.

Discussion

Lysozyme is inhibited by copolymers of glutamic acid and tyrosine, phenylalanine or leucine, to a much greater extent than by a homopolymer of glutamic acid. Thus, the efficiency of *p*(Tyr,Glu; 1:1) which was reported previously for the inhibition of ribonuclease (Sela, 1962), is an example of a more general phenomenon in which certain nonpolar amino acids may be substituted for tyrosine in the polyanionic inhibitor and the basic enzyme ribonuclease may be replaced by lysozyme.

Electrostatic interaction seems necessary for the inhibitory action, as the inhibitor molecules must be negatively charged. A copolymer of tyrosine and lysine exhibited essentially no inhibitory effect. The electrostatic interaction of the basic enzyme and the acidic inhibitor is also apparent from the ionic strength dependence of the inhibition as well as from the reversal of inhibition by the basic polyelectrolyte polylysine. It seems, therefore, that the type of inhibition described is not due to specific interaction with the catalytically active center of the enzyme, but rather results from the interaction of oppositely charged inhibitor and enzyme. Other positively charged enzymes would be expected to be similarly inhibited by acidic copolymers of the type used in this study.

As the great increase in the inhibitory efficiency of the copolymers investigated as compared to polyglutamic acid occurs when leucine, tyrosine, or phenylalanine are included in the inhibitor molecule, it seems resonable to assume that this additional effect is due to hydrophobic forces in which the side-chains of these amino acids participate. It was also shown that a mixture of free tyrosine and free glutamic acid has no inhibitory effect. Further, copolymers of tyrosine and glutamic acid containing only a small percentage of tyrosine, or a mixture of free tyrosine and polyglutamic acid, are no better lysozyme inhibitors than polyglutamic acid alone. Obviously the inhibitor in this system must be of a polymeric character, and its effectiveness in inhibition is markedly increased by the presence in the molecule of clusters of amino acids with hydrophobic side-chains. Similar observations were reported for the inhibition of the enzymatic activity of ribonuclease (Sela, 1962) and of the immune reaction between some polypeptidyl gelatins and their respective antibodies (Sela and Arnon, 1960).

The inhibition of lysozyme by the copolymers described seems to be related to the formation of small aggregates of the enzyme with the acidic macromolecules, as indicated by the centrifugation experiments. The *p*H dependence of inhibition may also be correlated with these aggregation and disaggregation phenomena. While *p*(Tyr,Glu; 1:1) had little inhibitory effect on ribonuclease at *I*:*E* = 10 above *p*H 6 (Sela, 1962), it fully inhibited lysozyme at the same ratio of inhibitor to enzyme throughout the *p*H range investigated (*p*H 4.5 to 9.0). However, at *I*:*E* = 1, lysozyme inhibition became incomplete above *p*H 6, suggesting that, also in the case of lysozyme, an increase in *p*H above 6 results in a decrease of inhibitory efficiency. The dependence of inhibition on *p*H is probably due to changes in the ionization of the various groups on the enzyme. It is unlikely that ionization of the inhibitor plays a role, as the copolymer contains only carboxyl groups whose ionization should result in a strengthening of the electrostatic bonds with the enzyme.

The inhibitory effect on lysozyme of the copolymers studied decreases dramatically in urea solutions. As urea acts on complexes of the enzyme both with a copolymer of glutamic acid and tyrosine and with a copolymer of glutamic acid and leucine, it is apparent that the action of urea involves ultimately the breaking of some hydrophobic bonds, resulting in disaggregation of enzyme and inhibitor molecules. It is possible that the effect of urea is an indirect one, namely, that it affects primarily other bonds in the enzyme or inhibitor, as a result of which the hydrophobic bonds between the molecules are weakened. On the other hand, the possibility that urea has a small direct effect on hydrophobic bonds has been mentioned by Kauzmann (1959). Steiner and Edelhoch (1962) have also suggested recently that detergents and urea attack similar types of internal linkage.

The finding that the cooperative effect of electrostatic and hydrophobic bonds results in an increased efficiency of enzymatic inhibition is similar to the observation of Bichowsky-Slomnicki *et al.* (1956) that, while basic amino acid residues are required for antibacterial activities of synthetic copolymers as well as of gramicidin S, the presence of leucine residues greatly enhances these activities.

Many natural inhibitors have a narrow enzyme specificity. Some macromolecular inhibitors, however, act in a less specific manner. Synthetic copolymers of amino acids have served in this study as model compounds for elucidating some of the features of such interactions.

Note Added in Proof:

Recent experiments have shown that *p*(Glu,Ala; 2:1), *n* 200, obtained kindly from Dr. M. Rigbi, is not a better inhibitor of lysozyme than *p*Glu. This is in agreement with our assumption that the increase in the efficiency of inhibition described in this paper is due to the presence of strongly hydrophobic side-chains in the inhibitory copolymers.

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Structural Studies of Ribonuclease. VII. Chymotryptic Hydrolysis of Ribonuclease A at Elevated Temperatures*

JOHN A. RUPLEY† AND HAROLD A. SCHERAGA

From the Department of Chemistry, Cornell University, Ithaca, New York

Received December 3, 1962

Ribonuclease A undergoes a transition at elevated temperatures which renders certain peptide bonds accessible to chymotryptic hydrolysis, leading to the production of large intermediates in the proteolytic reaction. These intermediates have been isolated and the sites of chymotryptic attack have been identified as tyr-cys (25–26), met-ser (79–80), tyr-lys (97–98), leu-thr (35–36), tyr-ser (76–77), phe-val (46–47), and an unidentified bond whose *N*-terminal member is glutamic acid or glutamine. The data suggest that in the thermal transition three regions of the ribonuclease polypeptide chain unfold, around residues 73 to 80, around half-cystine 26, and around tyrosine 97. The *C*-terminal region of the molecule, including tyrosine 115, probably is not affected by the thermal unfolding. Several other structural implications of the data are discussed, and the effects of solvent and temperature changes on the proteolytic reaction are considered.

Recent work in this laboratory and elsewhere (Harrington and Schellman, 1956; Hermans and Scheraga, 1961a,b; Tanford and Weber, 1959 [private communication]; Holcomb and Van Holde, 1962) on the reversible thermal transition of ribonuclease indicates that the native molecule is only partially unfolded in the transition. This paper presents experiments locating the portions of the polypeptide chain which unfold; these experiments are based on the hypothesis that the unfolded parts of a polypeptide chain are more susceptible than folded regions to attack by proteolytic enzymes (Mihalyi and Harrington, 1959). The results of experiments utilizing chymotrypsin as the proteolytic enzyme are described below; the use of trypsin will be discussed in the accompanying paper (Ooi *et al.*, 1963).

In order to relate the sites of chymotryptic hydrolysis to the unfolding of the molecule, several sets of facts must be established. First, the proteolysis must be clearly associated with the unfolding reaction; in this connection, both chymotrypsin and trypsin fail to hydrolyze native ribonuclease A at an appreciable rate (Spackman *et al.*, 1960; Scheraga and Rupley, 1962) but, as shown below and in the following paper (Ooi *et al.*, 1963), these enzymes rapidly hydrolyze the thermally unfolded molecule. Second, large intermediates in the proteolysis, in which only one or a few bonds have been hydrolyzed, must be isolated; if proteolysis is too extensive it is not possible to distinguish bonds broken as a result of their exposure in the transition from bonds broken at random (except for the requirements of enzymic specificity). Finally, the position of the splits must be determined by chemical analysis of the large intermediates isolated, a procedure greatly facilitated by the recent description of the amino acid sequence of ribonuclease A (Hirs *et al.*, 1960; Spackman *et al.*, 1960; Smyth *et al.*, 1962; Potts *et al.*, 1962; Gross and Witkop, 1962; Smyth *et al.*, 1963). A discussion of previous work on the limited proteolysis of ribonuclease may be found in a review by Scheraga and Rupley (1962).

*This work was supported by a research grant (E-1473) from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Public Health Service, and by a research grant (GB-75) from the National Science Foundation. It was presented before the Division of Biological Chemistry at the 140th meeting of the American Chemical Society, Chicago, September, 1961. Preliminary communications of some of this work have appeared (Rupley and Scheraga, 1960; Rupley *et al.*, 1962).

† Postdoctoral Fellow of the U. S. Public Health Service, National Cancer Institute, 1959–61. Present address: Department of Chemistry, University of Arizona, Tucson.

EXPERIMENTAL

Materials.—Ribonuclease was purchased as the crystalline material from Sigma and from Wilson Chemical Companies. The ribonuclease A fraction was obtained by chromatography of the crystalline protein on unsieved Amberlite IRC-50, XE-64 in 0.2 M sodium phosphate buffer, *p*H 6.47 (Hirs *et al.*, 1953), 7.5 × 60 cm column, and was deionized by passage through the mixed-bed ion exchanger, MB-1 (Rohm and Haas Co.) 5.0 × 40 cm column.

Ribonucleic acid (RNA) was purchased from Nutritional Biochemicals Co. and was used without further purification.

Diisopropylfluorophosphate was obtained as a gift from B. J. Jandorf of the Army Chemical Center, Maryland, and was used as a 0.1 M solution in dry isopropanol.

Acetyltyrosine ethyl ester and carbobenzoxyglycylphenylalanine were products of Mann Research Laboratories.