Strong Binding of Hydrophobic Anions by Bovine Serum Albumin Peptides Covalently Linked to Lysozyme*

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ABSTRACT: The small-peptide fraction, derived from a limited chymotryptic digestion of bovine serum albumin, and representing nearly 20% of the total protein weight, contains the strong hydrophobic anion binding sites of intact albumin. When these small peptides are covalently attached to lysozyme, used as a macromolecular support, binding of 1-anilinonaphthalene-8-sulfonate is only half an order of magnitude weaker than binding by native bovine serum albumin. In contrast, a lysozyme control, lysozyme derivatives containing peptides obtained from the macromolecular fraction of digested albumin, and the free small-peptide fraction have very low binding affinities for the same ligand.

In a previous paper (Jonas and Weber, 1970) we described the isolation, from a chymotryptic digest of BSA,[1] of a heterogeneous small-peptide fraction (av mol wt <2000) whose properties suggested a possible association with the strong hydrophobic anion binding sites of BSA. The SP fraction had a high relative content of basic (mainly arginine) and nonpolar amino acid residues (including one to two tryptophans), in agreement with reports that binding of hydrophobic anions to BSA requires a nonpolar environment and cationic charges on the protein (Klotz, 1953; Weber and Laurence, 1954), and occurs in the vicinity of tryptophan residues (Herskovits and Laskowski, 1962; Polet and Steinhardt, 1968; Swaney and Klotz, 1970).

Subsequently, we showed (Jonas and Weber, 1971) that chemical modification of arginine residues in BSA causes a marked decrease in the binding affinity of the protein for ANS, a typical hydrophobic anion.

Although all the available data pointed to a connection between the SP fraction and the strong hydrophobic anion binding sites of BSA, direct evidence for strong binding of ANS by this fraction could not be obtained. Addition of excess SP fraction to a digest of BSA increased slightly the binding ability of the digest for ANS, but SP fraction free in solution had an extremely low binding affinity for the ligand (Jonas and Weber, 1970).

In this work we describe in some detail the preparation and properties of the SP fraction. Also, we show that when the SP-fraction peptides are covalently linked to a macromolecular structure (lysozyme), which itself does not exhibit strong ANS binding, the affinity for ANS, accompanied by increase in fluorescence yield, is restored to levels approaching those of native BSA.

Materials and Methods

Crystalline BSA, lot no. F 71703, was purchased from Armour Pharmaceutical Co.; it was used without any treatment for the removal of dimer or fatty acids. Lysozyme (twice crystallized) and α-chymotrypsin were obtained from Worthington Biochemical Corp. 1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-*p*-toluenesulfonate ("Morpho CD1"), the water-soluble carbodiimide reagent, was purchased from Aldrich Chemical Co. The magnesium salt of ANS was prepared by the method of Weber and Young (1964). All common chemicals were commercial reagent grade materials, used without further purification, with the exception of urea which was recrystallized and deionized prior to use in the reduction and carboxymethylation of the macromolecular fraction of BSA. Deionized water was used throughout these experiments.

A Metrohm automatic titrator and pH-Stat, Model Combi-Titrator 3D, was used in following the chymotryptic digestion of BSA and of the macromolecular fraction of BSA. Column fractions were collected with a Research Specialties, Model 1205 D3, automatic fraction collector; amino acid analyses were carried out in Beckman Model 120C and Beckman-Spinco automatic amino acid analyzers. For the high-voltage paper electrophoresis a Beckman high-voltage power supply and a water-cooled tank containing a high flash point, immiscible organic solvent were used. Absorbance values were measured with a Zeiss PMQ II spectrophotometer. Fluorescence intensities were measured with the instrument described by Weber and Young (1964), using front face optics and printing out the ratio of fluorescence signal to exciting light intensity with a Dana digital voltmeter, Model 5403.

Preparation of SP Fraction. Citraconylation and limited chymotryptic digestion of the expanded BSA, at pH 7.7, have already been described (Jonas and Weber, 1970). After enzymatic cleavage of eight to nine peptide bonds and acid treatment of the digest for the inactivation of α-chymotrypsin and removal of citraconyl groups, the digest was lyophilized.

The best separation of macromolecular fragments from small peptides was attained by passing the BSA digest, dissolved in H_2O to give a 3% protein solution (more concentrated solutions were too viscous), through a Bio-Gel P-30 column (2.2 × 85 cm) equilibrated with H_2O. A typical elution pattern is shown in Figure 1.

Citraconyl residues were eluted with the SP fraction under

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[1] Abbreviations used are: BSA, bovine serum albumin; SP fraction, small-peptide fraction derived from limited chymotryptic digestion of BSA; ANS, 1-anilinonaphthalene-8-sulfonate; SP region, region of BSA where the SP fraction originates.

the second uv absorbance peak and had to be removed in a separate desalting step.

Amberlite IR-120 resin was first washed with H_2O for 24 hr, then it was packed into a 1.7 × 21 cm column. SP fraction plus citraconyl residues were applied to the column in 10 ml of 0.1 N HCl. After application of the sample the column was washed with about 200 ml of H_2O, followed by 4 N NH_4OH. Ammonia effluents were collected in 10–12-ml fractions, and absorbance at 280 mμ was measured. Fractions under the absorbance peak were pooled and lyophilized until all traces of ammonia were removed.

Preparation of Control Peptides from the Macromolecular Fraction of BSA. Material under the first peak eluted from the Bio-Gel P-30 column, containing approximately 80% of the total weight of BSA in macromolecular fragments, was lyophilized prior to reduction and carboxymethylation. Reduction and carboxymethylation were performed according to Crestfield *et al.* (1963) in 12-ml centrifuge tubes sealed with serological stoppers. After reaction, the solutions were dialyzed against six changes of H_2O, for 48 hr; precipitate formed gradually.

Following lyophilization, the reduced and carboxymethylated material was dissolved in a dilute ammonia solution (pH 8.5) to give a 1% protein concentration. Extensive digestion with α-chymotrypsin was carried out in the pH-Stat, at room temperature, maintaining the pH at 8.5 with ammonia. A total of 5 mg of α-chymotrypsin was used to digest 150 mg of protein, 3 mg being added at the beginning and 2 mg after 1.5 hr. Uptake of ammonia ceased 3–4 hr from the start of the reaction indicating that the digestion was nearly complete. The average size of the resulting peptides was estimated to be about 8 amino acid residues with an average molecular weight near 1000. The preparation was lyophilized and kept at −5° until use.

Carbodiimide-Coupling Reactions. A water-soluble carbodiimide, 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-*p*-toluenesulfonate (Goodfriend *et al.*, 1964), was used for the coupling of the SP fraction and control peptides with lysozyme in amide linkages. In each case, 2 ml of 2% lysozyme in H_2O was mixed with 20 mg of peptide and 40 mg of carbodiimide. After stirring 5 hr at room temperature, another 20 mg of peptide and 40 mg of carbodiimide were added to the original mixtures. Stirring was continued for 20 more hr in covered vessels. A control reaction of lysozyme alone with carbodiimide was run simultaneously. Precipitate formed in each of the reaction mixtures within 0.5 hr from the beginning of the reaction.

The products of the reaction were dialyzed for 48 hr against several changes of 0.05 M sodium phosphate buffer (pH 7.0) in order to remove excess reagents and low molecular weight products. The suspensions of modified lysozyme in phosphate buffer were used directly in the ANS binding experiments.

Amino Acid Analyses. Amino acids were determined by automatic analysis of desalted samples which had been hydrolyzed for 24 hr at 105° in 5.7 N HCl, in the presence of 0.05% mercaptoacetic acid. Cysteine and cystine were determined as cysteic acid after oxidation of samples with performic acid (Moore, 1963) followed by acid hydrolysis. Tryptophan was determined by the colorimetric method of Spies and Chambers (1949).

Peptide Mapping. Peptide maps of the SP fraction of BSA were obtained following the procedures described by Bennett (1967). High-voltage electrophoresis (2000 V for 2 hr) was performed first, followed by descending paper chromatography in butanol–water–acetic acid (4:5:1, v/v), with the organic phase as the mobile and the aqueous phase as the stationary media. Ninhydrin, Ehrlich, and Sakaguchi stains for peptides, tryptophans, and arginines, respectively, were prepared and applied as described by Bennett (1967).

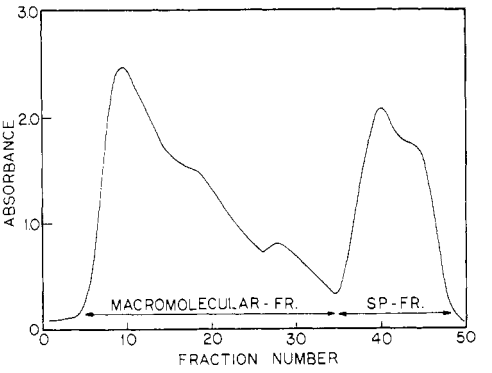

FIGURE 1: Fractionation of chymotryptic digest of BSA on Bio-Gel, P-30 column. Absorbance measured at 280 mμ; column equilibrated and eluted with water.

Binding of ANS. Binding of ANS by modified lysozyme and lysozyme control is represented graphically using the logarithmic expression derived by Klotz (1953) for the binding of ligands to n protein sites, having the same K_d and no interactions

$$\log \frac{n - r}{r} = -\log D + \log K_d$$

where n is the total number of binding sites per mole of protein, r moles of bound ligand per mole of protein, D molar concentration of free ligand, and K_d intrinsic dissociation constant.

The use of fluorescence parameters, especially fluorescence intensity changes, in binding studies has been described by Weber and Young (1964), Daniel and Weber (1966), and Jonas and Weber (1970).

In the present study the fluorescence yield of ANS bound to protein was more than 100 times higher than for free ANS; therefore the assumption that fluorescence was entirely due to bound ligand was justified. Binding experiments were carried out in 0.1 M sodium phosphate buffer (pH 7.0) by adding small increments of the modified lysozyme suspensions to an ANS solution. The change in ligand concentration upon protein addition was not significant. After each addition (10 μl of protein), the contents of the fluorescence cuvet were carefully mixed and the fluorescence intensity was measured at 470 mμ, using 345-mμ exciting light and 5-mμ bandwidths. F_0, the fluorescence of totally bound ligand, which could not be determined experimentally, was obtained by extrapolating a plot of $1/F$ *vs.* $1/P_0$ to $1/P_0 = 0$ (F, fluorescence intensity, and P_0, total protein concentration) (Weber and Young, 1964).

The effect of turbidity of the suspensions on the measured fluorescence was not significant. There was no contribution of scattered exciting light as indicated by a normal protein-bound ANS fluorescence emission spectrum (wavelength of maximum fluorescence at 472 mμ). Scattered fluorescence light did not contribute more than 3% to the fluorescence signal under the instrumental conditions used in our experiments. This estimate was obtained by observing fluorescence

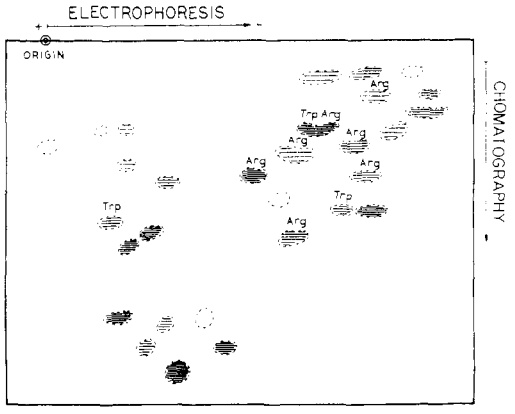

FIGURE 2: Peptide map of SP fraction. First dimension: high-voltage electrophoresis (2000 V, 2 hr) in pyridine–acetic acid–H_2O (1:10:89, v/v) buffer (pH 3.5). Second dimension: descending paper chromatography in butanol–H_2O–acetic acid (4:5:1, v/v). Spacing of horizontal lines across spots indicates relative ninhydrin color intensity.

changes in a quinine sulfate solution as a Celite suspension was added.

Direct quantum yield determinations at different r values were not performed; however, F_0 values extrapolated from F signals obtained at various r's had the same relative fluorescence as standard quinine sulfate solutions of the same absorbance at the exciting wavelength—an indication that the ANS quantum yield is constant for all binding sites.

TABLE I: Amino Acid Composition of SP Fraction and Macromolecular Fraction of BSA.[a]

Amino Acid	SP Fraction	Macromolecular Fraction	Total[b]	BSA[c]
Asp	3.92	10.06	8.83	9.37
Thr	3.25	7.34	6.52	5.66
Ser	5.87	4.28	4.59	4.60
Glu	12.01	14.30	13.84	13.20
Pro	3.10	6.00	5.42	4.95
Gly	3.85	2.62	2.87	2.83
Ala	10.79	7.30	8.00	8.14
$^1/_2$-Cys	1.03	6.48	5.39	6.01
Val	5.33	5.85	5.75	6.18
Met	0.34	0.98	0.85	0.76
Ile	2.91	2.02	2.20	2.30
Leu	12.84	9.99	10.56	10.80
Tyr	9.35	2.30	3.71	3.33
Phe	6.24	4.08	4.51	4.60
His	3.00	2.74	2.79	3.01
Lys	7.67	9.56	9.18	9.90
Arg	8.04	2.89	3.92	3.89
Trp	1.40	0.07	0.34	0.35

[a] Given as amino acid residues per 100 residues determined. Average of three preparations. [b] Sum of per cent amino acid compositions: 20% SP fraction plus 80% macromolecular fraction. [c] Calculated from the data of Peters and Hawn (1967).

Results

Small-Peptide Fraction of BSA (SP Fraction). Separation of the macromolecular from the SP fraction of BSA after limited chymotryptic digestion is shown in Figure 1. Protein content was determined by Folin's method (Folin and Ciocalteau, 1927) using intact BSA as the standard. The SP fraction (four preparations) represents from 17 to 23% of the protein weight, while the macromolecular fraction accounts for the rest.

Amino acid analyses for both fractions are given in Table I. Differences in per cent content of polar and hydrophobic amino acids (Nozaki and Tanford, 1971) between the two fractions, and each fraction and BSA, are apparent. The SP fraction is cationic with respect to BSA, and has a higher relative content of hydrophobic residues, including one or both tryptophans, but no disulfide bridges. A more detailed discussion of the amino acid distribution in BSA has been given in a previous paper (Jonas and Weber, 1970).

Peptide maps of the SP fraction were obtained in an attempt to estimate the number and size of the constituent peptides. Figure 2 shows a typical map. Three different preparations gave almost identical results.

Specific stains for tryptophan and arginine reveal the presence of tryptophan in three peptides, and arginine in at least seven. Most arginine-containing peptides have a high relative mobility in the electrophoresis system, an indication of a high cationic charge and possibly small size. There are from 10 to 15 strong ninhydrin-staining spots on the maps, and 10 to 15 faint spots; thus the average molecular weight of the peptides is around 800.

Lysozyme Coupled with BSA Peptides. The approximate extent of the coupling reaction was estimated from amino acid analysis of the reaction products (Table II). The per cent isoleucine residue content which is low in the BSA peptides (2.9 and 2.0% in SP fraction and control peptides, respectively) and somewhat higher in lysozyme (4.2%) was used as the normalizing factor. An ideal amino acid residue for this type of calculation would be one whose relative content in lysozyme is high, while it is zero in the peptides. In the present case the contribution of isoleucine from the conjugated peptides to the total isoleucine content of lysozyme derivatives is not negligible. Assuming a 20–30% incorporation of new amino acids (based on a 20% yield of the carbodiimide peptide macromolecule coupling reaction, estimated from yields of a peptide–peptide-coupling reaction), the isoleucine contribution from the peptides to the lysozyme derivatives is about 0.7%. Excess amino acids/100 amino acid residues of lysozyme were calculated as follows: lysozyme derivative % aa $\times$ (lysozyme % Ile/lysozyme derivative % Ile $-$ 0.7%) $-$ lysozyme % aa. Calculations using methionine instead of isoleucine content gave similar results. Excess amino acids present in the lysozyme–peptide derivatives, estimated in this manner, resemble qualitatively the amino acid composition of the SP fraction and macromolecular fraction of BSA. The SP fraction–lysozyme derivative has about 30% excess amino acids when compared with the lysozyme control, while the control peptide–lysozyme derivative has nearly 35% more amino acids. Thus, there are on the average four peptides coupled to each lysozyme molecule.

Extent of lysozyme polymerization during the carbodiimide-coupling reaction was not determined, but possible effects on the binding of ANS were accounted for by using the lysozyme control, which had been exposed to carbodiimide in the absence of peptides.

TABLE II: Amino Acid Composition of Lysozyme and Lysozyme–Peptide Derivatives.[a]

Amino Acid	Lysozyme	Lysozyme–SP Fraction	Excess[b] Amino Acid	Lysozyme–Control Peptides	Excess[c] Amino Acid
Asp	16.67	15.66	3.7	16.38	5.8
Thr	7.80	7.51	1.9	6.42	1.0
Ser	8.24	9.65	4.3	6.88	1.2
Glu	4.41	5.85	3.2	8.07	6.6
Pro	2.19	2.29	0.8	5.21	4.9
Gly	10.02	10.06	3.8	7.65	0.5
Ala	9.71	9.90	3.2	9.36	3.1
½-Cys	4.45	3.47	0.05	4.22	1.3
Val	4.45	4.54	1.5	4.22	1.3
Met	1.63	1.44	0.2	1.39	0.3
Ile	4.15	3.88	0.8	3.76	0.7
Leu	6.49	7.49	3.3	7.73	4.1
Tyr	2.47	2.75	1.1	2.09	0.4
Phe	2.30	3.16	1.8	2.60	1.3
His	0.91	0.97	0.4	1.42	1.0
Lys	4.32	3.67	0.5	5.89	3.8
Arg	8.61	8.40	2.3	6.17	0

[a] Expressed as amino acid residues per 100 residues. [b] Difference between per cent lysozyme–SP-fraction residues multiplied by a factor of 1.30 (obtained as described in the text) and per cent lysozyme residues. [c] Difference between lysozyme–control peptide amino acid content multiplied by a factor of 1.35 (see text) and lysozyme residues.

ANS Binding. Figure 3 summarizes the binding data for lysozyme and lysozyme–BSA peptide derivatives with ANS. A theoretical binding curve for three ANS sites having an intrinsic dissociation constant identical with that of ANS bound to BSA, under the same experimental conditions ($K_d = 2 \times 10^{-6}$ M) (Daniel and Weber, 1966), is included for comparison with our results.

Lysozyme control and lysozyme–control peptide derivatives have very low binding affinities for ANS as measured by fluorescence intensity changes. No binding of ANS by the free SP fraction can be observed by the fluorescence technique. The lysozyme–SP-fraction derivative, on the other hand, binds ANS with an affinity only half an order of magnitude lower than native BSA; it has on the average 2.7 binding sites/modified lysozyme molecule with a K_d of 1.3×10^{-5} M.

Discussion

The remarkable capacity of the lysozyme–SP-fraction derivative to bind ANS, in contrast to the low binding affinity of the lysozyme–control peptide derivative and lysozyme control for the same anion, confirm our supposition that the strong hydrophobic anion binding sites are located in the SP fraction of BSA.

The SP fraction appears to originate from expanded BSA about one-third from the amino-terminal end of the protein, taken in a linear amino acid sequence (Jonas and Weber, 1970). A high local concentration of basic residues in this region of intact BSA explains numerous experimental observations that BSA binds hydrophobic anions in preference to similar cations even when the overall charge on the protein is negative. The SP region of BSA also contains 30% of the most hydrophobic residues in the protein (Trp, Phe, Tyr, Leu, Val, and Met) (Nozaki and Tanford, 1971) and 40% of all aromatic amino acids. Since there are no disulfide bridges in the SP fraction, it seems that hydrophobic interactions contribute significantly in maintaining the compact structure of the SP region in native BSA.

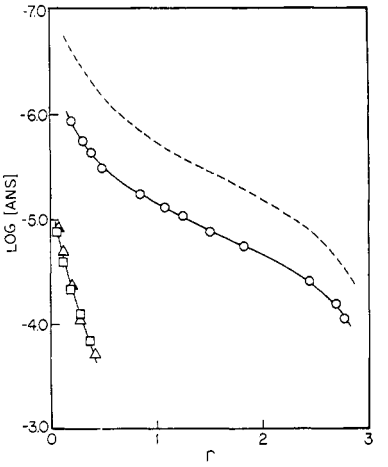

FIGURE 3: Binding curves of ANS by lysozyme–BSA-peptide derivatives in 0.1 M sodium phosphate buffer (pH 7.0). Plots of log [ANS] *vs.* *r* for: (○) lysozyme–SP-fraction derivative; (△) lysozyme–control peptide derivative; (□) lysozyme control. (– –) Theoretical curve for three binding sites with $K_d = 2 \times 10^{-6}$ M.

Although there is no direct evidence that arginine residues contribute the positive charge in the binding of hydrophobic anions by BSA, they are strongly implicated by their high relative concentration in the SP region of BSA, and by the effect that their chemical modification has on the binding of anions such as ANS. Lysine modification does not seem to affect the binding properties of BSA, while chemical reaction of arginines decreases the binding capacity by one to two orders of magnitude (Jonas and Weber, 1971).

Binding of ANS by the free SP fraction could not be detected by the fluorescence technique. Interactions between dye and SP fraction do not occur in the aqueous environment at the concentrations used in the binding experiments (approximately 10^{-4} M). Ion-pair formation is precluded by the low concentrations of SP fraction and ANS, and by the presence of 0.2 N buffer. Hydrophobic interactions (Kauzmann, 1959), which in the case of ANS are manifested by a large increase in quantum yield accompanied by a blue shift in fluorescence (Weber and Laurence, 1954), are absent. Specific interactions between aromatic groups in the peptides and ANS are not expected to occur at 10^{-4} M concentrations. Even in well-known cases of complex formation as in the intramolecular complexes of FAD and NADH, interactions between the aromatic rings require spatial proximity (covalent linkage) or high concentrations of the interacting groups after the covalent bond is broken ($>10^{-3}$ M) (Weber, 1970).

When the SP fraction is covalently attached to the surface of lysozyme there is a marked increase in the fluorescence of ANS on binding. A shift in the fluorescence maximum to 472 mμ also indicates hydrophobic interactions. The peptides are apparently removed from extensive contact with water by interaction with nonpolar groups on the surface of lysozyme.

In this manner a hydrophobic environment analogous to that found in native BSA is possibly generated.

Studies of macromolecule–small molecule interactions on synthetic polymers by Klotz and coworkers (Klotz *et al.*, 1969; Klotz and Harris, 1971) have shown that linear polymers have a much lower binding ability for hydrophobic anions (methyl orange) than highly cross-linked or branched polymers. These authors attribute the difference in behavior to the difference in conformation, the "open and extended" conformation of the linear polymers being less favorable than the "compact locally concentrated" conformation of the other polymers or of native BSA. In view of our results we may add that some secondary interactions promoted by the proximity of nonpolar groups may be essential in binding processes involving hydrophobic interactions.

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Lactobacillus plantarum Exoribonuclease. Effect of Urea Treatment on the Processive Mode of Degradation*

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ABSTRACT: The effect of urea on *Lactobacillus plantarum* exoribonuclease (a processive nuclease) has been tested. Although dilute urea has no effect on enzyme activity, treatment with 1.67 M urea leads to a biphasic, logarithmic loss of activity. This loss of activity is accompanied by decreases in both the K_m and V_{max} over an order of magnitude but by no change in the ionic requirements of the enzyme. More importantly the residual enzyme activity regardless of the prior urea treatment is always strictly processive with both synthetic polynucleotides and yeast RNA. Thus the factors conferring processivity are not affected by urea treatment.

Recently at least three bacterial enzymes have been shown to degrade single-stranded RNA processively. That is they degrade individual RNA strands essentially to completion before starting to degrade another RNA strand. These are polynucleotide phosphorylase from *Escherichia coli* (Thang *et al.*, 1967) and from *Micrococcus luteus* (Klee and Singer, 1968), ribonuclease II from *E. coli* (Nossal and Singer, 1968), and exoribonuclease from *Lactobacillus plantarum* (Logan and Singer, 1968). It is not known what chemical or structural features of those enzymes lead to processive degradation rather than the more common random degradation.

We have studied the effect of the general denaturing agent urea on the *L. plantarum* enzyme to see if processivity is lost under conditions leading to local denaturation of the protein. However under our experimental conditions which lead to extensive loss of activity and changes in the enzyme's kinetic parameters, the residual activity is always expressed as strictly processive degradation.

Materials and Methods

The growth of *L. plantarum* and the purification and assay of the *L. plantarum* exoribonuclease have been described previously (Logan and Singer, 1968; Gardonio and Logan, 1971). Tris·HCl buffer (pH 8.6) was used instead of 2-amino-2-methyl-1,3-propanediol buffer (pH 8.6). A fraction III enzyme preparation was used in all the experiments reported here except the gel electrophoresis experiments which were performed with fraction V enzyme.

[^{3}H]- and [^{14}C]poly(A) were prepared in our laboratory by standard techniques from [^{3}H]- and [^{14}C]ADP (Amersham–Searle and Schwarz BioResearch) and polynucleotide phosphorylase kindly supplied by Dr. M. F. Singer or purchased

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