Biochemistry

Volume 10, Number 6 March 16, 1971

Macromolecule–Small Molecule Interactions. Strong Binding by Intramolecularly Cross-Linked Polylysine*

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ABSTRACT: Polylysine has been thiolated with thiobutyrolactone and the SH groups introduced have been oxidized to produce S–S cross-linkages in the polypeptide. In solutions of high ionic strength and at pH's of 8–9 the introduction of S–S linkages endows these polypeptides with substantial binding ability toward small anions. These polypeptides should thus provide a suitable matrix for conjunction of binding groups and potential catalytic sites.

Combination of a small molecule with a biologically active protein is generally the initiating step in a sequence of biochemical transformations. For this reason the nature of binding interactions has long been a subject of interest. In early studies of complexes of serum albumin with small anions it became apparent that apolar interactions, as well as electrostatic ones, are involved in binding (Klotz, 1946). In essence the protein provides clusters of apolar side chains (Swaney and Klotz, 1970) accessible from bulk solvent, which could interact with apolar parts of the small molecule.

On this basis one would expect to be able to generate binding ability for small molecules in synthetic polymers containing apolar residues. Many water-soluble polymers do bind small molecules, but linear polymers do not have an avidity comparable to that of the native protein serum albumin (Klotz and Shikama, 1968; Klotz and Sloniewsky, 1968).

A possible reason for this difference in behavior may lie in the difference in conformation (Figure 1), a synthetic polymer having an extended very open conformation, in contrast to the compact locally concentrated conformation of serum albumin. Polyvinylpyrrolidone, for example, which binds small anions about a third as extensively as serum albumin, has an intrinsic viscosity, $[\eta]$, of 22 (ml/g) as compared to 4 for the protein. Such hydrodynamic behavior clearly shows that the synthetic macromolecule has a much more open, or less compact, conformation in aqueous solution.

Two different avenues seem available for increasing the local concentration of residues provided by a linear polymer. One approach would be to grow branches along the main polymer chain and thereby to increase the local density of residues. An example of this course is provided by polyethylenimine,[1] apolar derivatives of which have been shown to possess remarkable binding affinities (Klotz *et al.*, 1969). A second approach would be to expose the linear polymer to environmental conditions which lead to a more compact conformation and then to lock it into this steric arrangement with covalent cross-linkages. An example of this course is provided in this investigation. Polylysine in a partially shrunken state has been frozen into a relatively compact conformation by the introduction of disulfide bonds. This modified polypeptide shows a substantial increase in its ability to bind small molecules.

Experimental Section

Polylysine hydrobromide was purchased from Pilot Chemicals, Inc. (lot 90, mol wt 100,000). The bromide ion was replaced by acetate as follows (Klotz and Shikama, 1968). Polylysine hydrobromide dissolved in 150 ml of acetate buffer (pH 6.57), 0.1 ionic strength, was dialyzed against 2 l. of buffer for 2 days with frequent changes of buffer solution. Dialysis was then carried out against distilled water, for 2 more days, to remove excess acetate. The solution was filtered and the polypeptide recovered by lyophilization.

Thiolation of polylysine was carried out at room temperature, by the following procedure. About 0.3–0.4 g of polypeptide (acetate) was dissolved in 10 ml of water, under nitrogen, and the pH was adjusted to 9. Thiobutyrolactone (purchased from Saber Laboratories, Morton Grove, Ill.) was added to the solution, and the reaction followed with a pH-

* From the Biochemistry Division, Department of Chemistry, Northwestern University, Evanston, Illinois 60201. *Received August 31, 1970.* This investigation was supported in part by a grant from the National Science Foundation. It was also assisted by support made available by a U. S. Public Health Service training grant (5T1-GM-626) from the National Institute of General Medical Sciences.

[1] Polylysine should also be able to provide a main trunk for generation of a branched polypeptide.

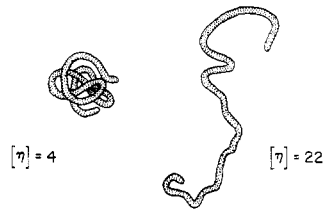

FIGURE 1: Representation of conformations of compact protein molecule, such as serum albumin, with an intrinsic viscosity $[\eta] = 4$ as compared to that of a very extended polymer, such as polyvinylpyrrolidone with $[\eta] = 22$.

Stat. The acid produced was simultaneously neutralized by the NaOH in the syringe of the pH-Stat. The reaction was complete in about 80 min. In the absence of polylysine, the thiobutyrolactone added was hydrolyzed to only a trivial extent (about 3% of added reagent) in the same period. The reaction mixture, adjusted to pH 7, was usually passed through an ion-exchange column (Dowex 1-X2, in the acetate form) under nitrogen to remove any hydrolyzed anionic γ-mercaptobutyrate ion.

The thiolated polylysine was then diluted with water or aqueous solvent to reduce the polypeptide concentration to about 0.03%. The pH was then adjusted to some value between pH 7 and 12 and the solution exposed to air for a period of 1–2 days. After this period the content of thiol groups was assayed by Ellman's method (Ellman, 1959). Even after 2 days exposure to air, a few per cent of the thiol groups were still unoxidized. These were alkylated, after the aqueous solution had been concentrated tenfold in a rotary evaporator and adjusted to pH 7.0, by addition of iodoacetamide (to a concentration less than 5% that of Lys residues). This mixture was dialyzed first against acetate buffer (pH 6.5) and then against water, and the resultant solution was lyophilized to isolate the oxidized thiolated polylysine.

Control samples of polylysine were taken through a similar series of steps except that thiobutyrolactone was omitted in the initial step. This material showed sedimentation behavior indistinguishable from untreated polylysine.

The disulfide content of the oxidized thiolated polylysine was determined by the analytical method of Zahler and Cleland (1968). In the presence of 0.7 mM dithiothreitol at pH 9, complete reduction of the disulfide groups was achieved within 15 min.

Samples of oxidized, thiolated polylysine containing from 8–30 residue % of modified Lys groups were prepared by these procedures.

The binding of methyl orange by polylysine and modified polylysines was measured by the equilibrium dialysis technique (Klotz *et al.*, 1946; Hughes and Klotz, 1956), in acetate buffer at pH 5.6 (Klotz and Shikama, 1968). The polypeptide concentration was 0.1%.

Circular dichroic spectra in the ultraviolet region were obtained with a Cary Model 60 spectropolarimeter equipped with a circular dichroism attachment. The polypeptide concentration was about 0.05–0.1%.

Sedimentation coefficients were measured with a Spinco Model E analytical ultracentrifuge, using schlieren optics. Polypeptide concentrations were about 0.5%. For slowly sedimenting samples rotor velocities of 67,770 rpm were used; for others, 59,780 rpm.

Viscosities were measured with an Ostwald viscometer having a flow time for water of about 100 sec.

TABLE I: Binding Constant k_1 for Formation of First Complex of Macromolecule and Methyl Orange.

Macromolecule	% Lys Residues Cross-Linked	$k_1 \times 10^{-5}$ [a]
Polylysine		0.08
S–S preparation A	10.5	0.2
B	10.5	0.2
C	10.5	0.37
D	13.7	0.46
E	13.7	0.4
F	13.7	1.45
Bovine serum albumin[b]		0.54
Polyvinylpyrrolidone[b]		0.22

[a] Measurements at 25° in acetate buffer (pH 5.6). [b] Taken from Klotz and Shikama (1968).

Results

To facilitate comparison the extent of binding (Figure 2) has been depicted in terms of the moles of bound small molecule per 10^5 g of polymer, r, as it varies with concentration of free (unbound) methyl orange, A. The results for unmodified polylysine agree well with previous determinations (Klotz and Shikama, 1968). The binding curves for preparations A and B (omitted from Figure 2) are not significantly different from that of unmodified polylysine. Derivative A was kept in an 0.01 Tris-cacodylate buffer at pH 7 containing 2.5 M glycine during oxidation of SH groups to S–S by air; derivative B was in the same buffer adjusted to pH 8 and containing 2 M NaCl. The first increase in binding was observed with derivative C, cross-linked in 0.01 M Tris buffer (pH 8) containing 1.5 M Na_2SO_4. Its extent of binding was comparable to that of preparation D (see Figure 2). The latter derivative was essentially a repeat preparation of C. Oxidation of a solution containing[2] less Na_2SO_4 (0.75 M) also led to a material, derivative E, with binding properties comparable to D. In contrast, a substantial increase in binding was observed with derivative F (Figure 2) in which the solution of 0.01 M Tris–1.5 M Na_2SO_4 was raised to pH 9 before oxidation was permitted to proceed. It is clear that alkaline pH is desirable. On the other hand, pH's appreciably higher than 9 led to insoluble derivatives.

The binding affinities can also be compared quantitatively in terms of the first binding constant, k_1 (Table I). This constant can be calculated conveniently by extrapolating values of r/A for different concentrations, to infinitely dilute solutions (Klotz and Urquhart, 1949).

Hydrodynamic properties of the polylysine derivatives are assembled in Table II. Circular dichroic spectra are shown in Figure 3.

Discussion

It is obvious that substantial increases in binding affinity can be created in polylysine upon introduction of disulfide cross-linkages. The first binding constant k_1 has been raised from 0.08×10^5 to 1.45×10^5 (Table I); that is, almost 20-fold in

[2] In one experiment, Na_2SO_4 was replaced by 0.9 M sodium citrate, but with the pH maintained at 8. This derivative (G) was also comparable to D in binding properties.

TABLE II: Hydrodynamic Properties of Disulfide Cross-Linked Polylysines.

Substance	Concn (%)	s_{20}[a] Obsd (S)	D_{20}[a] Obsd (cm²/sec), × 10⁷	$s/D \times 10^6$	$[\eta]^{a-c}$ (g/ml), at 25.0°
S–S preparation C	0.30	3.15	1.50	2.10	
D	0.40	2.75	1.54	1.79	10
F	0.34	2.96	1.47	2.01	
Polylysine					230

[a] Measurements in 0.1 M acetate buffer (pH 5.6). [b] A preparation that showed an $[\eta] = 77.5$ g/ml and $s_{12°}$ of 1.66 (at $C = 0.5\%$) and contained 9% of the Lys residues in S–S cross-linkages showed no enhanced binding ability. [c] For polylysine, an $[\eta]$ of 230 corresponds to a degree of polymerization of 900 (Wooley and Holzwarth, 1970).

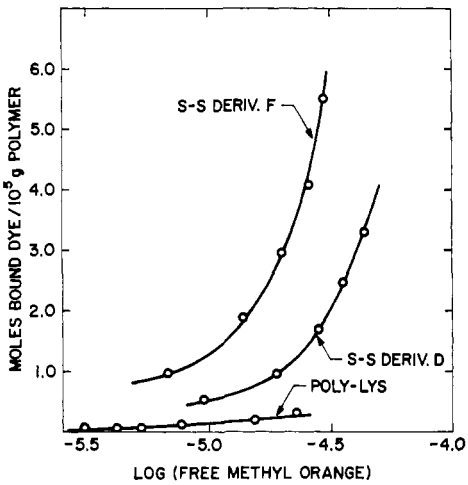

FIGURE 2: Extent of binding of methyl orange at pH 5.6 and 25° as a function of free (nonbound) dye concentration.

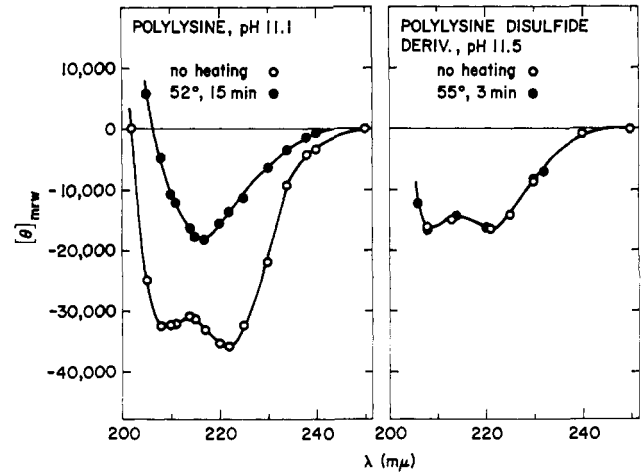

FIGURE 3: Circular dichroic spectra of unmodified polylysine (taken from Greenfield and Fasman, 1969) and of disulfide cross-linked polylysine, derivative C. Both heating conditions have been shown by Fasman and coworkers (Greenfield *et al.*, 1967; Lynn and Fasman, 1968) to produce β structure in unmodified poly-L-lysine.

going from unmodified polylysine to S–S derivative F. With the latter derivative, in fact, binding affinity is almost threefold greater than that of the native protein serum albumin which is the outstanding example of proteins with generalized binding potential.

At this stage one must consider the question, is the S–S polylysine *intramolecularly* or *intermolecularly* cross-linked?

As pointed out in the experimental section, oxidation was carried out with thiolated polylysine at concentrations of 0.03%. The polypeptide concentration thus was substantially lower than that found to lead to intramolecular cross-linking in reduced ribonuclease (White, 1960, 1961; Anfinsen and Haber, 1961; Epstein *et al.*, 1963) and hence should be dilute enough to lead to intramolecular S–S linkages in thiolated polylysine also. Direct evidence to this point is also provided by hydrodynamic properties (Table II). The intrinsic viscosity of S–S polylysine is markedly lower, 10 g ml^{-1}, than that of polylysine, 230 g ml^{-1} (Table II). This indicates strongly that the disulfide linkages have produced a more compact molecule. Similarly sedimentation coefficients (Table II) indicate that intramolecular cross-linking is responsible for the increase observed in binding. The ratio s/D for the derivatives C, D, and F is constant[3] (Table II), although the binding constant (Table I) increases three- to fourfold. The last of these derivatives was prepared at the highest practicable pH in the presence of concentrated salt, that is, under conditions that reduce electrostatic repulsions to a minimum (and still keep a soluble derivative), whereas the other two were cross-linked under conditions where electrostatic effects should favor more swollen conformations. Nevertheless the relative (apparent) molecular weights as judged from s/D are essentially the same. Derivative F, which should be the most compact of the three, does indeed show substantially stronger binding affinity (Figure 2, and Table I).

The number of disulfide linkages in derivative C corresponds to about 27/65,000 weight, that is, nearly twice as many as in serum albumin (17 S–S/65,000 mol wt). One might expect, therefore, that S–S polylysine should be locked into a relatively restricted conformation. Circular dichroic spectra[4] (Figure 3) indeed show that S–S polylysine does not change conformation on warming at pH 11. This is in marked contrast to unmodified polylysine which, as has been reported by others

[3] It was not feasible to obtain reliable diffusion or sedimentation coefficients for polylysine itself. Electrostatic effects plus the extended swollen conformation make s and D very low and difficult to evaluate at concentrations near 0.3%.

[4] The spectra of Figure 3 also indicate that the helix content of S–S polylysine is about half that of the unmodified polypeptide. A reduction in helix content would certainly be expected for the modified polylysine since the S–S groups (introduced at pH 9) will impose strong constraints on the polymer when it is exposed to the pH of 11 which normally favors complete conversion into a helical conformation.

(Davidson *et al.*, 1966; Sarkar and Doty, 1966; Lynn and Fasman, 1968), is transformed from an α-helical to a β-conformation at pH 11 if the temperature exceeds 50°.

The *N*-carboxyanhydride method of polymerization gives rise to relatively homogeneous polypeptides with a well-defined, sharp degree of polymerization (Tanford, 1961). The introduction of thiol groups on 10% of the residues followed by oxidation leads to an average of about 39 disulfide cross-linkages in a polymer of 10^5 mol wt. The larger the number of cross-links introduced the more nearly uniform should be the population of macromolecules at least in regard to compactness. It should be mentioned in this connection that schlieren photographs in sedimentation studies showed single, symmetric peaks.[5]

Local segments, however, within a given polypeptide or on different polypeptides, are likely to show variation in conformation and hence in affinity for small molecules. Thus on a given polypeptide there may be a distribution of sites with different affinities, as is found in proteins. On the other hand, in proteins, all macromolecules of a given species presumably are identical, whereas this is unlikely in these cross-linked polypeptides.

The affinity of cross-linked polylysine for methyl orange is not as great as that of acylated polyethylenimines, the highly branched strong-binding polymer described recently (Klotz *et al.*, 1969). This may be because the branched acylpolyethyleniminesa re more compact and provide higher local concentrations of apolar and cationic sites than do the S–S polylysines. The comparative spectra of bound methyl orange on the two classes of polymer tend to corroborate this explanation. The dye bound to acylpolyethylenimines (at low *r* values) shows an absorption peak near 430 nm, as it does also when complexed with serum albumin. In contrast, when bound to the cross-linked polypeptide the dye exhibits an absorption peak near 460 nm, close to that (465 nm) for methyl orange in a bulk water environment.

These S–S cross-linked polylysines should thus provide a relatively rigid framework for the attachment of additional apolar substituents. It should be possible, therefore, to generate clusters of apolar groups bracketed by cationic lysines, conditions which are very favorable for binding both in the natural protein serum albumin (Swaney and Klotz, 1970) and in synthetic polymers (Klotz *et al.*, 1969). Furthermore it should also be feasible to tie pendant catalytic groups to the cross-linked polypeptide. Thus these polypeptides should provide matrices for conjunction of neighboring binding and catalytic sites, the juxtaposition of which may lead to polymers with unusual catalytic properties.

[5] This should be a sensitive indicator of heterogeneity since the sedimentation coefficients of different preparations with different average percentages of cross-linkages are markedly different.

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