Protamine and Polyarginine Bacteriolysis. Similarities in Its Mechanism with Chromatin DNA Picnosis

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Protamine and polyarginine had bacteriolytic effects indicating their primary sites of action as being wall components and showing bacterial diversity genetically determined. Shake-incubation was required in producing cell-lysis. Studies on *Bacillus subtilis* revealed a high polycation multiplicity per cell in lytic event displaying multihit lysing kinetics; bacteriolysis was inhibited by trypsin, pronase, purified polyanionic wall polysaccharide, and by dissociative actions of salt hypermolarities used in isolation of nucleic acids. The inactivation of polycation lytic abilities during bacteriolysis was accompanied by modifications in electrophoretic running of protamine and polyarginine. It is suggested as mechanism of cell-lysis, the multiple zonal surface condensations of polyanionic wall components by basic polypeptides, likely similar with chromatin DNA picnosis. This analogy is discussed.

Introduction

The inhibition of genetic transformation in *B. subtilis* by protamine in sublethal concentration was assumed to be caused by interaction of the polycation with recipient wall polysaccharides [1] suggested as stabilizing material in the structure of DNA binding sites [2 – 4]. In this context, the finding of wall modifications induced by protamine or protamine-like polycations could be considered a further support for the above assumption.

Here we describe the bacteriolytic effects of both protamine in high concentrations and polyarginine in low doses as a proof that their primary site of action is the cell-wall. The inhibition of polycation bacteriolysis in different conditions used for dissociation of natural protein-DNA aggregates as well as the kinetic traits suggest a model of cell-lysis by multisite wall component condensations, analogous in its mechanism to DNA compression in eukaryotic chromatin or in sperm head. A provisional study of polycation bacteriolysis about its spread amongst six different genera showing heterogenous sensitivity, is also described.

Materials and Methods

1. Strains

B. subtilis strains WT and 861 [1] were used as principal subject-matter. The strains used in screening of polycation bacteriolysis were received from different Collections of Cantacuzino Institute Bucarest, as follows: (a) *B. anthracis* strain vaccin 1 and *B. subtilis ruber* (Dr. Maria Antohi); (b) *B. subtilis* var. *mesentericus*, *B. megaterium*, *Shigella dysenteriae* strains Type 1 and Type 2, *Sh. flexneri* – 5 strains and *Klebsiella pneumoniae* – 5 strains (Dr. Gabriela Boltasu); (c) *Staphylococcus aureus*, strain Oxford as well as 18 different strains used in the propagation of lysotyping bacteriophages (Dr. S. Iordanescu); (d) *Pseudomonas aeruginosa* – 36 different strains (Dr. Eugenia Meitert); (e) *Salmonella typhi* – 3 strains, *S. enteritidis* – 7 strains, *S. typhi murium* – 10 strains, *S. bovis morbificans* – 5 strains, *S. paratyphi* A – 2 strains, *S paratyphi* B – 3 strains and *S. jawa* – 5 strains (Dr. M. Negut) *.

2. Reagents, media, stock solutions and bacterial initial suspensions

Reagents. Poly-L-arginine sulfate MW 15,000 – 50,000 daltons, protamine sulfate MW 8,000 daltons and trypsin 2 × cryst. were from ICN-Pharmaceuticals-Life Sciences Group-Cleveland. Polyanionic purified Enterobacterial Common Antigen (ECA) was a gift of Dr. A. Marx (Cantacuzino Institute Bucarest). All other reagents were from Merck.

Media. Medium for competence [1] (abbreviated as MC) was used as basal medium for different conditions of the interactions between cells, polycations

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* All the strains used are continuously preserved in our laboratory.

and their inhibitory factors. Tryptose Blood Agar Base Difco (TBAB) and Penassay Broth Difco Medium 3 were routinely used for growth and preservation of the strains.

Stock solutions were performed as follows: Protamine 2 mg ml^{-1} in distilled water; polyarginine 400 µg ml^{-1} in 0.05 N HCl the pH of which was finally adjusted at 4.5 with NaOH 2 N; trypsin, pronase E and ECA were dissolved in MC, in separate solutions of 400 µg ml^{-1}; sodium chloride 2 M and sodium perchlorate 1 M in MC as separate solutions. The solutions were stored at 4 °C and used in a time interval of 3 days.

Bacterial initial suspensions having OD_{340} of 2.1 were prepared from cultures of 14 h at 37 °C on slant TBAB. The cells harvested and washed once in MC by centrifugation and resuspension, were spectrophotometrically quantified and immediately used.

3. Conditions of polycation bacteriolysis and its inhibiting using B. subtilis strains WT and 861

(a) *The lytic polycation concentrations, the importance of shake incubation and cell-lysis as function of time.* As Table I illustrates, MC, polycation stock solutions and bacteria were distributed in tubes of 18/180 mm obtaining different series of mixtures of protamine and polyarginine respectively, in ranges of concentrations mentioned in the Fig. 1 and bacteria at OD_{340} of 0.30. Polyarginine that gave fine precipitates in the samples with higher concentrations, was redissolved by shake incubation at 37 °C for 30 – 60 min before the addition of bacterial suspension. Separate series of polyarginine and protamine samples were incubated as follows: (i) Incubation at 37 °C for 60 min with reciprocal shaking at 130 strokes min^{-1} the sample tubes having slanted angle of 30°; (ii) unshaken incubation at 37 °C for 60, 120, and 180 min; (iii) shake incubation as above for different time intervals (Fig. 2).

Table I. Distribution of MC, polycation stock solutions and bacterial initial suspension (in ml) in different polycationic series of samples.

	Samples						
	1	2	3	4	5	6	7
MC	1.85	1.80	1.75	1.70	1.65	1.60	1.60
Polycation	0.05	0.10	0.15	0.20	0.25	0.30	–
Bacteria	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Distilled water	–	–	–	–	–	–	0.30

The bacteriolysis was estimated by two methods: (i) OD_{340} measurements at the ends of different incubations, using a Beckman Model 25 spectrophotometer and QS cells with 1 cm path length; (ii) phase contrast microscopy of different slides prepared from each sample and immediately examined. For all series there were polycation free control samples (Table I) comparatively examined by both methods.

(b) *Inactivation of lytic abilities of the polycations during cell-lysis.* In incubated clarified bacterial samples with protamine 200 µg ml^{-1} and polyarginine 40 µg ml^{-1} respectively, bacterial initial suspension in 0.1 ml portions was redistributed, reincubated with shaking at 37 °C for 60 min or more and cellular presence estimated as above.

(c) *Electrophoretic analysis of the polycations passed by a lytic cycle.* Bacterial initial suspension 0.3 ml, stock solutions of either protamine 0.3 ml, or polyarginine 0.5 ml were mixed in MC 1.4 ml and 1.2 ml respectively, performing 5 such mixtures for each polycation. Both series were shake-incubated at 37 °C for 60 min, collected in two separate Erlenmeyer flasks of 50 ml and kept overnight at 4 °C with magnetic stirring at 300 – 400 rotations per min. From each mixture an aliquot of 2 ml was mixed with 2 ml saccharose 2 M and 0.1 ml glacial acetic acid, and was then layered as 0.6 ml portions in tubes with polyacrylamide gels (initial acrylamide 10%). The preparation of gels and the running were performed according to Johns [5] except sample running time that was 1 h. The staining and destaining were carried out according to Weber and Osborn [6]. Comparative bacteria free samples of polycations were in parallel prepared as above.

(d) *Inhibition of cell-lysis by proteolytic and polyanionic pretreatments of the polycations, and by salt hypermolarities.* In different series of 1.5 ml aliquots of MC stock solutions of polycations, proteolytic enzymes and ECA polyanion were separately added as follows: protamine 0.2 ml; polyarginine 0.2 ml; trypsin, pronase E and ECA, each in 0.2 ml portions from intermediary adequate dilutions in MC to perform ranges of concentrations (Fig. 3), in separate protamine and polyarginine series corresponding to each of them. All series were shake-incubated at 37 °C for 10 min and initial bacterial suspension 0.1 ml per sample was then distributed.

Different 1.7 ml aliquots of NaCl 2 M and $NaClO_4$ 1 M were completed with 0.2 ml of stock solutions either of protamine or of polyarginine, and with 0.1 ml of bacterial initial suspension. In order to stop bacteriolysis already started, in samples with protamine 200 μg ml^{-1} and polyarginine 40 μg ml^{-1} (Table I) shake-incubated for 5 min, salt crystals of NaCl and $NaClO_4$ corresponding to 2 M and 1 M respectively, in different samples were quickly added and continously incubated. All series with factors taken as inhibitors (trypsin, pronase E, polyanionic ECA, salt hypermolarity) were shake-incubated at 37 °C for 60 min and finally estimated for cell lysis.

4. Conditions for assaying polycation bacteriolsysis in other bacteria

Other strains, except those of *Staphylococcus*, in the same conditions described for *B. subtilis* strains WT and 861 (Table I) were assayed using shake incubation, OD_{340} and microscopic examinations but preparing only samples with protamine 200 μg ml^{-1} and polyarginine 40 μg ml^{-1}. In working with staphylococci, MC was replaced with a mixture Penassay Broth/MC in ratio 1/5.

Results and Discussion

1. The bacteriolysis, a result of multihit cell-polycation interactions; its bacterial diversity genetically stable

Both basic polypeptides, salmon sperm protamine and polyarginine had lytic effects on strains WT and 861 of *B. subtilis* without strain differences. Revealed by OD_{340} measurements they started significantly with concentrations of protamine 100 μg ml^{-1} and polyarginine 20 μg ml^{-1}, the higher concentrations with the same 5/1 ratio having different decreasing values of turbidity (Fig. 1) and showing clear-cut stronger effect of the polyarginine. The cell-lysis appeared only in shake condition of incubation since identical series of protamine and polyarginine statically incubated had little or no significant decrease of OD_{340} values (Fig. 1) even if the incubation periods were 2 – 3 times longer. In the slides carried out from shaken clarified polyarginine samples there were very rare coccoid granules, free or in small clumps. In unshaken turbid samples, frequent free immobile and shape altered granulated bacteria forming agglutinates at higher polyarginine concentrations were observed. Similar, more attenuated morphological alterations were exhibited by protamine series at higher doses.

Since the different kinds of bacteriolysis, obligatorily involved wall modifications [7, 8], the lysis of *B. subtilis* cells by protamine in higher concentrations and by protamine-like polycation polyarginine, constitutes a support of the assumption that inhibition of genetic transformation by protamine previously described [1] has as primary site of interactions the wall components of recipient cells.

As Fig. 1 illustrates, the minimal concentrations with highest lytic effects were 200 μg ml^{-1} protamine and 40 μg ml^{-1} polyarginine. In clarified incubated samples with these concentrations, the readdition of bacterial cells followed by shake-reincubation was not accompanied by bacteriolysis, showing the involvment of almost all polycation molecules in the interactions of the first lysing incubation. These polycation concentrations corresponded to 1.5×10^{16} protamine molecules ml^{-1} and to 7.4×10^{14} polyarginine molecules ml^{-1}. Since the starting OD_{340} of 0.30 corresponded to about 6×10^7 cells ml^{-1} (counted as colony forming units) then the bacteriolysis occured at the ratios of about 2.5×10^8 protamine molecules/cell and 2.2×10^7 polyarginine molecules/cell (in considering the average value of molecular weight of the reagent used). Albeit the polycation multiplicities per cell were very high, the lytic events signifi-

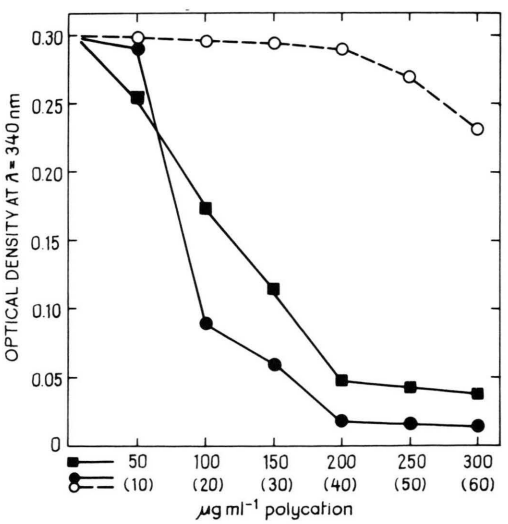

Fig. 1. Bacteriolysis exerted by protamine and polyarginine on *B. subtilis* strain WT, as a function of polycation concentrations; incubation time was 60 min. Protamine shake incubated series (■–■). Polyarginine static incubated series (○- - -○), and shake incubated series (●–●). Protamine static incubated series gave a plateau of OD_{340} values of 0.30 for all concentrations. Figures in brakets = polyarginine concentrations.

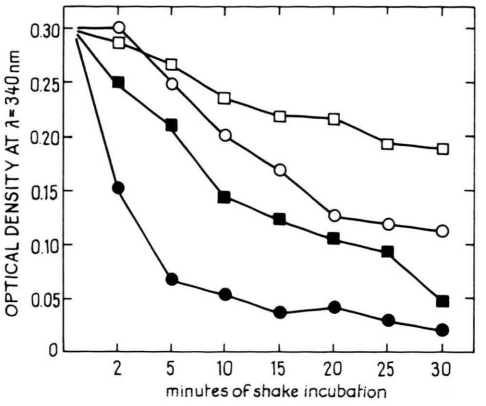

Fig. 2. Decreases of OD_{340} values of bacterial-polycation mixtures as a function of shake incubation time. The plotted lines illustrate OD_{340} values of separate sampled series of either protamine 100 μg ml^{-1} (□–□) and 200 μg ml^{-1} (■–■) or polyarginine 20 μg ml^{-1} (○–○) and 40 μg ml^{-1} (●–●).

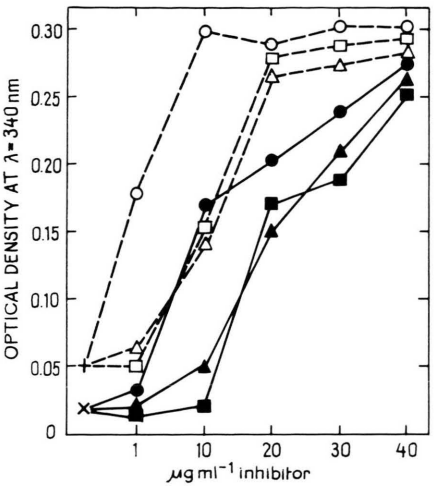

Fig. 3. Inhibitory effects of trypsin (○) (●), pronase E (□) (■) and polyanionic Kunin's antigen (△) (▲) on protamine 200 μg ml^{-1} (dashed lines) and polyarginine 40 μg ml^{-1} (continuous lines) lytic actions. OD_{340} values given by control inhibitor free samples of protamine (+) and of polyarginine (×) simultaneously shake incubated, were taken as start points of plotting. *B. subtilis* strain WT was used.

cantly occured merely in shake condition of incubation suggesting the requirement of increasing frequency of efficient lytic hits besides the hit frequency ensured by high molecular density. Plotting the OD_{340} values of protamine and polyarginine bacterial samples against the time of shake-incubation (Fig. 2), the decrease of turbidities occured progressively in 25 – 30 min showing, together with the requirement of shaking, an apparent multihit trait for the lytic event. However, comparing the polycation lysing kinetics with single hit killing kinetics, *e. g.* described for some colicins [9], the trait of multihit kinetics for the first seems to be clear.

The bacteriolysis described in this report appears to be the culmination of polycation damaging effects on bacteria including bacteriostatic and lytic free bactericidal action [10, 11], and membrane alterations [12, 13]. In *B. subtilis*, protamine exhibits a dose dependent gradient of its effects: with concentrations of 10 – 20 μg ml^{-1} it inhibits genetic transformation and lytic action of lysozyme [1]; lytic free bactericidal effect with 30 – 80 μg ml^{-1}; bacteriolysis with higher 100 – 300 μg ml^{-1} doses. The delay in observing polycation bacteriolysis can be explained by differences in many respects including the polycations used, the experimental conditions (particularly the shake-incubation) and the bacteria involved. The last difference resulted from the diversity in polycation bacteriolysis exhibited by the 108 strains belonging to six different genera challenged in this work. Polyarginine exerted cell-lysis on all assayed strains of *Staphylococcus*, *B. subtilis* var. *mesentericus* and strain *ruber*, *B. megaterium*, two strains of *Pseudomonas* (strains EM 4 and EM 5) and the two strains of *Sh. dysenteriae*. From these polyarginine lysing strains only those of *B. subtilis* and *Pseudomonas* were lytic by protamine action. The other strains of *Pseudomonas*, *Sh. flexneri*, *Salmonella*, *Klebsiella* and *B. anthracis* were resistant to both polycations. The sensitivity to lysis as well as the resistance were unchanged during 100 passages of *B. subtilis* strains WT and 168, and 20 passages of the other strains, suggesting genetic stability. Many assays with *B. subtilis* WT including a total population of about 5×10^9 cells in obtaining resistant nonlytic mutants by simple selection using polyarginine as selective pressor, were unsuccessful.

2. Inhibition of polycation bacteriolysis by trypsin and pronase pretreatments

As Fig. 3 shows, trypsin and pronase E pretreatments of polycations induced a dose dependent inhibition of bacteriolysis. The proteolytic inhibition

was significantly higher with protamine suggesting that the cleavage number per molecule required for inactivation was smaller for protamine than that required for polyarginine inactivation. The lower proteolytic inactivation rate of polyarginine together with its higher lytic activity (Figs 1 and 2) suggest the importance of polypeptide length in bacteriolysis because polyarginine polypeptide is 2 to 6 times longer than that of protamine. In the experimental conditions of this work it seems that the longer was the basic polypeptide the stronger was its lytic ability.

3. Inhibition of polycation bacteriolysis by polyanionic wall polysaccharide-ECA and by dissociative agents used in nucleic acid isolation

Since cell-lysis itself indicated the wall as bearing the sites of polycation actions and assuming salt binding associations as the nature of polycation-cell surface interactions, we assayed the possible inhibitory effects on bacteriolysis either of anionic polysaccharide described by Kunin [14] or of the agents used in the dissociations of anionic nucleic acid-polycationic protein aggregations [15 – 17]. Revealed by OD_{340} measurements and by microscopic examination, both dissociative agents (salt hypermolarities) and polyanionic lipopolysaccharide (Fig. 3) were evident inhibitors of bacteriolysis. Moreover, sodium chloride or sodium perchlorate added after 5 min of incubation of the polycation-bacterial mixtures, stopped cell lysis inducing a hypermolarity rescue of the cells not yet lysed.

4. The inactivation of polycation lytic ability during a bacteriolytic period of 60 min

The readdition of bacteria in clarified samples shake incubated for a first period of 60 min, was not followed by lysis during a second shake incubated interval of 60 min, both protamine and polyarginine being inactivated in the first period. These inactivations were apparently the consequence of the modifications induced in the polycation molecules during lytic interactions since, revealed by polyacrylamide gel electrophoresis, the principal band of protamine underwent significant alterations, exhibited by a pattern of 3 bands (Fig. 4). In its turn, the polyarginine homogenous region given by molecular weight distribution of polypeptides between 15,000 and 50,000 daltons became lightly striped having a tight band in the faster zone after a lytic period. The new

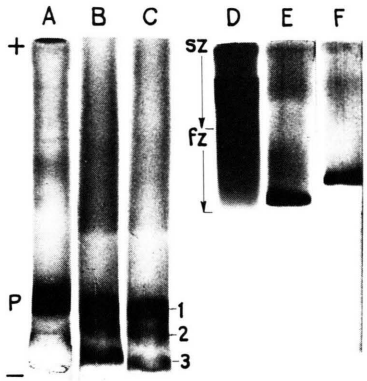

Fig. 4. Changes in the electrophoretic pattern of polycations following bacteriolytic actions. Lanes A and D = control bacteria free samples of protamine and polyarginine respectively. Protamine (lanes B, C) and polyarginine (lanes E, F) in bacterial samples after cell lysis. 1, 2, 3 = bands resulted from principal band (p) of protamine. *sz* and *fz*-polyarginine slow zone and fast zone of the electrophoretic region.

bands, certainly were provided by altered polycations, because with started bacterial concentration, the cell proteins resulted from lysis were not enough to give such dense bands.

5. A possible model of multisite polycation-wall condensation analogous to chromatin DNA package, as trigger mechanism of the cell lysis

The bacteriolysis by protamine and polyarginine exhibits some molecular traits apparently different from those of the other types previously described [7, 18 – 21]. The high polycation multiplicity per cell in lytic event, the inactivation of polycations during cell-lysis, the salt hypermolarity rescue of the cells to the started bacteriolysis, the alteration of electrophoretic pattern of the polycations engaged in lytic interactions, suggest together the direct involvement of polycation molecules in the lytic events. In this context, the indirect inducible polycation action on some endogenous lytic factors as those described previously [7, 22], seems highly unlikely.

The apparently direct participation of protamine and polyarginine in cell-lysis, the role of protamine in condensing DNA in sperm head [23] and the "largo sensu" configurational analogy between reiterative anionic groups of wall polysaccharides (teichoic acids [24] and lipopolysaccharides [25]), and repetitive phospho-groups of the double stranded DNA, lead us to suggest for polycation bacteriolysis a mo-

del of multisite polycation-wall condensation analogous to chromatin DNA picnosis exerted by histone octamers [26, 27]. Considering bacterial envelope as a multilayer assembly stabilized by polyanionic wall polysaccharides [8, 28], and having continuous supramolecular allosterism [29], the model assumes that polycation chains interact with polyanionic wall components stabilizing ranges progressively more extensive of ionic bonds, inducing multizonal condensing areas, resulting in wall splits and triggering cell-lysis. The condensing complexes would be delivered being revealed electrophoretically as altered polycationic patterns (Fig. 4). Analogically, the condensing ratio of the wall-polyanions involved in polycation aggregations could be near to packing ratio of 6.8/1 of the DNA in chromatin, ensured by octamerized histone polycations and evaluated by Thomas and Kornberg [27]. Such a condensing ratio would account for deleterious splits induced in bacterial envelope.

The model apparently match the findings described in this work, as follows: (i) Much more lysing strains amongst Gram positive bacteria richer in more exposed polyanionic heteroglycoside macromolecules than amongst Gram negative organisms with multilayered capped lipopolysaccharides [8, 28]; (ii) high polycation multiplicity per cell in lytic event as a requisite for multisite condensation; (iii) the requirement of shake incubation increasing the probability of efficient hits in the progressing of condensing salt binding ranges; (iv) the inhibition of bacteriolysis by purified polyanionic polysaccharide able to compete the cell sites in polycation-wall salt binding; (v) the inhibition of lysis by dissociative agents used in nucleic acid isolation able to impede polycation salt binding; (vi) the higher lytic efficiency of polyarginine molecules with longer ranges of cationic active groups similarly more active in supposed condensing mechanism as arginine rich histone H3 and H4 fractions display the central role in nucleosome DNA packing [30]. The higher involvement of polyarginine longer chains in assumed wall condensation mechanism would account for significant decrease of staining material in the slow zone of electrophoretic region corresponding to polypeptide fractions with higher molecular weights (Fig. 4). The last fractions would be engaged in condensing complexes at a level able to change their cationic type of electrophoretic running. Since shorter polypeptides were less engaged in condensation binding they persisted in cationic running, forming a dense band in the faster zone.

However, in spite of the resistance exhibited by Gram negative bacteria, the sensitivity to polycation bacteriolysis of a few strains of *Shigella* and *Pseudomonas* could be explained by higher diversity in the more complex structure of cell envelope of these organisms [8, 31].

Judging polycation bacteriolysis by the concept of comparative molecular physiology well illustrated by Reissig [29], the model of polycation-wall condensation seems not to be conceptually isolated from the present view (*e. g.* [32, 33]) on the natural polycation action in polyanionic macromolecule condensation, as the DNA package in chromatin or in sperm head.

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