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STIMULATION OF *BACILLUS SUBTILIS* MEMBRANE ADENOSINE TRIPHOSPHATASE BY CATIONIC BACTERICIDAL AGENTS

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Summary

The environmental Mg^{2+} used in preparation of *Bacillus subtilis* membranes was found to influence the responses of the associated ATPase to cetyltrimethylammonium bromide (CTAB). Membranes prepared using fluids containing higher Mg^{2+} levels exhibited lower control activity than was seen with low Mg^{2+} membranes. Increased environmental Mg^{2+} resulted in higher stimulations with lower doses of the agent. ATPase of all three membrane types was stimulated in two concentration ranges, but in the doses tested, CTAB inhibited the ATPase of only those membranes obtained using fluids containing high Mg^{2+} for every stage of the isolation. Sonication of membranes for 25 s at half maximum output yielded three fractions, consisting of a soluble form which was sensitive to CTAB stimulation at 25 μg/ml of assay mixture; small, 95—110 nm, vesicles, which were resistant to CTAB at 25, 75, and 150 μg/ml, and large vesicles, similar to untreated membranes both in morphology and responses to detergent. Combinations of detergent and protein (β-lysin or arginine-rich histone) produced activity appearing to be additive when the protein level was present in a high concentration and the detergent was present at levels corresponding to the minimum influence. Mixtures of a maximally stimulating dose (75 or 100 μg/ml) of detergent and a small amount of protein produced activities that were at least 92% or more of the expected sums of individual stimulations. Interference occurred with the following mixtures: high amounts of detergent and protein; high protein and 10 or 15 μg/ml CTAB; and β-lysin and arginine-rich histone, both at high levels. These data are consistent with a hypothesis that the two peaks in CTAB stimulation reflect two adjacent ATPase sites, one of which is also susceptible to stimulation by cationic protein.

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Abbreviation: CTAB, cetyltrimethylammonium bromide.

Introduction

Fresh sera from certain species (rat, rabbit, horse, man) are bactericidal for a number of Gram-positive microorganisms [1, 2]. The agent responsible was termed β-lysin by Pettersson [1] to distinguish it from the serum antibody-complement bactericidal system, known then as α-lysin. An accidental discovery that serum filtered through asbestos-cellulose filter pads was non-bactericidal led to the development of a filtration-elution procedure [3,4] which resulted in an approximate 300-fold purification and 4-fold concentration of the agent. That it is a cationic protein is indicated by observations that loss of activity occurs with tryptic digestion [5], and incubation with nucleic acids [6] or anionic phospholipids [5,7].

Lytic studies with cells, protoplasts, and cell walls suggested that the cytoplasmic membrane is the principal target for killing [8,9]. As the initial effort in elucidating its mode of action, the influences of β-lysin, arginine- and lysine-rich histones, cetyltrimethylammonium bromide (CTAB), protamine sulfate, tetra- and diamine compounds, and phospholipase A on membrane adenosine triphosphatase (ATPase) of *Bacillus subtilis* ATCC 6633 were compared [10]. The cationic bactericidal proteins and CTAB stimulated the membrane-associated enzyme of *Bacillus subtilis*, but had little influence on a form of ATPase obtained by sonication followed by centrifugation at 39 000 × *g*.

Variations in the influences of CTAB and β-lysin have been observed. The levels of ATPase induced by β-lysin have been similar for different membrane preparations when enzyme activity is expressed in unit values. However, the control enzyme levels vary, thus affecting the degrees of stimulation observed, when the latter is expressed in percent values. Membrane age could not be correlated in a consistent manner with the fluctuations in responses to cationic agents (unpublished findings). The role played by Mg^{2+} in maintaining structural and functional integrity of bacterial membranes was first emphasized by Weibull [11]. It seemed reasonable to determine if Mg^{2+} might influence the observed stimulations by cationic agents.

CTAB was selected for a Mg^{2+} study because of the complex nature of the response of membrane ATPase to this agent [10]. In addition, the present study includes information on the solubility properties of the sonicated ATPase forms, their behavior in the presence of CTAB at low as well as high dose levels, and on the number of sites for action by the cationic proteins and the detergent in producing observed stimulation of enzyme activity.

Materials and Methods

The sources of all reagents have been given in previous reports from this laboratory as were the procedures for preparation of β-lysin and enzyme and protein assays [10,12]. A unit of enzyme activity is defined as the amount catalyzing the release of 1 nmol of P_i under the assay conditions.

Membranes were obtained by eight procedures designated A through H (Type C corresponds to that used in previous work [12]). Protoplasts were formed by addition of lysozyme (100 μg/ml) to cells suspended in 50 mM Tris · HCl buffer, pH 7.2, containing 200 mM sucrose and $MgCl_2$ (10 mM in the cases

of A—D; 20 mM for E—H membranes) followed by centrifugation. The protoplast pellets were suspended in shocking fluid, 50 ml of cold Tris · HCl buffer containing $MgCl_2$ at 10 mM (A, E), 5 mM (B, F), 0.5 mM (C, G), and 0.3 mM (D, H) concentration. All membrane types were collected after deoxyribonuclease treatment and were washed twice with 50-ml portions of 50 mM Tris · HCl buffer containing 5 mM $MgCl_2$. Use of the latter buffer fluid for storage of all membrane types facilitated getting the appropriate Mg^{2+} concentration in the assay [12]. Membranes of the C type were used for studying the solublility properties of the sonicated ATPase and the number of sites for action.

Sonication of membranes was carried out as described previously [10] for 25 s. The larger membrane fragments were removed by centrifugation at 46 800 × *g* for 30 min and were reconstituted with Tris · HCl buffer to the original volume. The supernatant fluid was subjected to centrifugation at 109 000 × *g* for 1 h at 2°C. After removal of the fluid, a small pellet was evident, and it was resuspended in Tris · HCl buffer at 1/8 the original volume. 0.2 ml of the resuspended membrane pellets and of the supernatant fluids were assayed for ATPase in the presence of 0, 25, 75, or 150 μg/ml CTAB. For comparison of the different sonicated fractions, results are expressed in nmol P_i · released $min^{-1} \cdot mg^{-1}$ protein. Negative stains of the ultracentrifuged pellet were prepared as described previously [9].

Results

Variability in responses of membranes to cationic agents and role of Mg^{2+}

A more detailed study of membranes whose response to CTAB does not fit that which we have reported reveals two peaks of stimulation of ATPase which occur at higher doses of detergent (Fig. 1) than seen in the previous work [10]. Stimulation occurring at about 50 μg/ml appears to correspond to the inhibition noted previously since this level marks the minimum between the two stimulation ranges. To enable statistical comparisons to be made, the data presented below is expressed in units of enzyme activity.

Table I summaries the control and the CTAB-induced levels of ATPase of A, B, C, D, E, F, G, and H membrane types prepared using fluids varying in Mg^{2+}

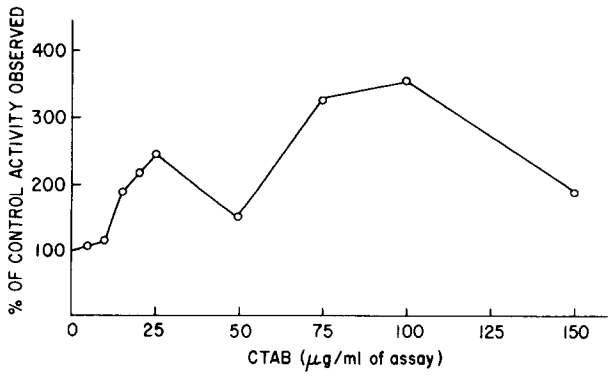

Fig. 1. CTAB-induced stimulation of *B. subtilis* membrane ATPase prepared according to the original procedure or as designated in this report, C type.

concentration as stated previously. The concentrations of detergent marking the positions of the two peaks and of the minimum influence between them are included in the table. An inverse correlation can be seen between activity observed and the environmental Mg^{2+} used for osmotic shocking of the protoplasts (Stage 3). The lowest levels were seen with membranes of the A type where the Mg^{2+} level was 10 mM during all but the last stage of preparation. Detergent-induced inhibition of enzyme (printed in italics) was seen most consistently with membranes prepared in the presence of 10 or 20 mM Mg^{2+} at Stages 1 and 2, and 10 mM at Stage 3. Lower Mg^{2+} during Stage 3 also resulted in a shift to higher levels of the first peak of stimulation (10—15 μg/ml) as well as a similar shift in the minimum influence by CTAB (15—25 μg/ml).

The complete data for CTAB responses of all eight membrane types was subjected to a statistical analysis using the Full Factorial Analysis model with all factors being random. Factor A has two levels: 10, 10; and 20, 20 . . .; Factor B has four levels: 10, 5, 0.5 and 0.3; and Factor C has eleven levels: 0 through 150 μg of CTAB. Factor A was shown to have a significant difference ($P < 0.05$) between its two levels. Factor B showed a significant difference ($P < 0.01$) among its four levels, as did Factor C among its eleven levels. Interaction

TABLE I

CONTROL AND CTAB-INDUCED ATPase ACTIVITIES ASSOCIATED WITH *BACILLUS SUBTILIS* MEMBRANES ISOLATED USING FLUIDS CONTAINING VARIABLE AMOUNTS OF Mg^{2+}

The sequence of numbers indicate the millimolar concentration of Mg^{2+} in: (1) fluid used to wash harvested cells, (2) stabilizing medium for protoplast formation, (3) osmotic shocking fluid, (4) fluid used to wash and store membranes.

Environmental Mg^{2+} at stages (1,2,3,4)	Type of membrane	Units of ATPase observed in the						
		Absence of CTAB (control)	Presence of CTAB in the following regions and dose levels					
			1st peak	μg of agent per ml	Minimum influence *	μg of agent per ml	2nd peak	μg of agent per ml
10, 10, 10, 5	A	290	774	10	*150.2*	20	1520	75
		250	715	10	*168*	20	1340	75
10, 10, 5, 5	B	310	916	10	*264.2*	15	1790	75
		310	735	10	*219*	20	1625	75
10, 10, 0.5, 5	C	361	949	15	*335*	25	2115	75
		425	1160	15	438	25	2115	75
10, 10, 0.3, 5	D	374	1085	15	*335*	25	2020	75
		387	1110	15	477	25	2060	75
20, 20, 10, 5	E	335	890	10	*297*	25	1755	75
		361	1000	15	*322*	20	1760	75
20, 20, 5, 5	F	322	852	15	335	25	1945	75
		306	1032	15	*322*	25	1935	75
20, 20, 0.5, 5	G	452	1110	15	452	25	2050	75
		458	1050	15	503	25	2125	75
20, 20, 0.3, 5	H	452	1148	20	580	25	2275	75
		400	1160	20	568	25	2330	75

* CTAB-induced inhibition, that is, activities lower than control levels, are printed in italics.

was detected between A and B, A and C ($P < 0.05$), and between B and C ($P < 0.01$). Factor ABC indicated interaction among factors ($P < 0.01$). It is concluded that a relationship (of a non-linear type) exists between environmental Mg^{2+} during preparation of membranes and their enzyme responses to CTAB.

Characterization of a sonicated form of ATPase with respect to solubility properties and susceptibility to stimulation by cationic agents

Fig. 2 depicts the control activities and activities due to CTAB (25, 75, and 150 μg/ml) associated with the sediment (circles) and supernatant (triangles) fractions obtained following low speed (46 800 × *g* for 30 min, open symbols) and high speed (109 000 × *g* for 1 h, closed symbols) centrifugations of sonicated membranes. Control activity was demonstrable in all fractions. The sonicate ATPase consisted of two forms, one sedimentable at 109 000 × *g* and the other, soluble. The CTAB effects on the low speed pellet, which consisted of large membrane vesicles, when examined in the electron microscope, were similar to those reported for unsonicated membranes [10]. Consistent with the preliminary work [10], the low speed supernatant and ultracentrifuged fractions were not susceptible to stimulation by 75 μg/ml CTAB. The effects of 25 μg/ml on these three fractions were strikingly different. The activity of the high speed pellet was completely suppressed. On the other hand, the low speed supernatant and soluble ATPase forms were stimulated 2—3-fold by 25 μg/ml. The latter findings are compatible with the earlier work with sonicated fractions since 25 μg/ml was not tested previously [10]. The fraction which was sedimented at 109 000 × *g* was, therefore, the only form found to be totally resistant to CTAB. Negative staining of this fraction revealed membrane vesicles approx. 95—110 nm in diameter (not shown).

The number of sites for action by cationic agents

Table II summarizes the results obtained with 25 and 75 μg/ml CTAB alone

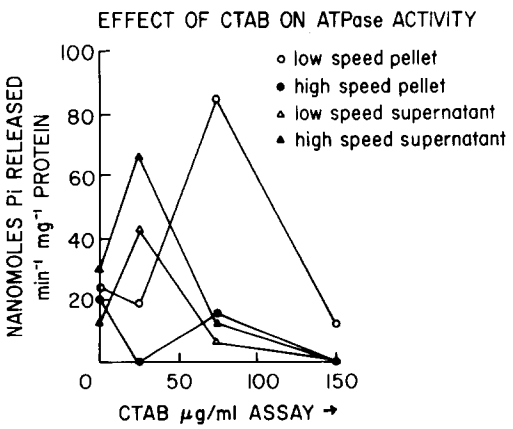

Fig. 2. ATPase levels of sonicated membrane fractions when tested alone and in the presence of CTAB. Membranes were sonicated in glass vials held in ice using the medium probe of a Biosonic III sonifier at one-half maximal output for 25 s. Aliquots were centrifuged at 46 800 × *g* for 1 h at 2°C. 0.2 ml of the resuspended membrane pellets and of the supernatant fluids were assayed: ○, low speed pellet; •, high speed pellet; ▵, low speed supernatant; and ▲, high speed supernatant.

TABLE II

ATPase STIMULATIONS INDUCED BY MIXTURES OF β-LYSIN PLUS 25—100 μg/ml CTAB

The response to CTAB of membranes used in this experiment was such that maximum stimulation occurred with 75 μg/ml, and inhibition occurred in the presence of 25 μg/ml. Values suggesting that additive stimulations had occurred are italicized. Units of bacericidal activity per ml of the assay. One unit is equivalent to 2.7 μg of protein.

Test	Control activity (units)	Agent 1	Activity observed in the presence of Agent 1 (units)	Agent	Activity observed in the presence of Agent 2 (units)	Activity in the presence of Agents 1 + 2 (units)	
						Observed	Expected *
1	607	β-lysin, 64 units/ml	813	CTAB, 75 μg/ml	1722	1555	2110
2	607	β-lysin, 8 units/ml	672	CTAB, 75 μg/ml	1722	1955	1969
3a	607	β-lysin,	813	CTAB,	432	*1280*	820
3b	477	64 units/ml	781	25 μg/ml	329	*990*	776

* Expected values were calculated using the formula, $E = A + B + xC$, where $x = -0.7$. E, expected ATPase activity; A, ATPase activity with β-lysin; B, ATPase activity with CTAB; C, control ATPase activity.

and in combination with β-lysin. The data on mixtures of protein and an optimal stimulating dose of CTAB (75 μg/ml) are the top two rows in the table and that for a mixture of protein and an inhibiting level of CTAB is found below these lines. In order to determine the best expected value to fit the observed data, a statistical model was formulated and evaluated using students's t-statistic. The 95% confidence interval for the parameter x in the model $E = A + B + xC$ is (—1.75, 0.35). E, expected ATPase activity; A, ATPase activity with β-lysin; B, ATPase activity with CTAB; and C, control ATPase activity. The t-statistic is minimized when x is equal to —0.7.

Interference was seen in Test 1 where both agents were present in high amounts. On the other hand, activity seen with a mixture of a low level of β-lysin and the optimally stimulating concentration of detergent was >99% (Test 2) of the expected value. The mixture of a high amount of protein and an inhibiting CTAB dose when present alone (Tests 3a and 3b) permitted activity greater than the expected values. This experiment was repeated using membranes responding to detergent in a manner similar to that shown in Fig. 1. Additive action was restricted narrowly to a combination of a high level of β-lysin and detergent at its minimum influence dose (not shown).

Table III presents data for mixtures of arginine-rich histone and CTAB using membranes of the second type. It can be seen that a wider range of histone levels acted in an additive manner with 50 μg/ml CTAB. It was not possible to demonstrate additive actions between very low CTAB levels and high protein. In fact, 5 μg CTAB/ml actually depressed the independent arginine-rich histone or β-lysin actions at any concentration of protein agent.

Combinations of the two cationic proteins produced activities close to values that would be expected when both agents were present at suboptimal stimulat-

TABLE III

ATPase STIMULATIONS INDUCED BY MIXTURES OF ARGININE-RICH HISTONE PLUS 50 AND 100 μg/ml CTAB

Response of membranes used in this experiment differed substantially in that 100 μg/ml was the optimum dose and 50 μg/ml marked the minimum influence. Values suggesting that additive stimulations had occurred are italicized.

Test	Control activity (units)	Agent 2	Activity observed in the presence of Agent 1	Agent 2	Activity observed in the presence of Agent 2	Activity in the presence of Agents 1 + 2 (units)	
						Observed	Expected *
1	529	Arg-His, 20 μg/ml	555	CTAB, 100 μg/ml	2020	2095	2205
2	484	Arg-His, 100 μg/ml	557	CTAB, 50 μg/ml	645	*1220*	863
3	484	Arg-His, 125 μg/ml	660	CTAB, 50 μg/ml	645	*1130*	966
4	484	Arg-His, 150 μg/ml	800	CTAB, 50 μg/ml	645	*1200*	1107

* Expected values were calculated by the same formula used for Table II.

ing levels. Interference occurred when one or both agents were present at maximally stimulating doses.

Discussion

The fluctuations observed in influences of each cationic agent on membrane-associated ATPase were characteristics of the membranes, since the responses were reproducible using the same preparation of membranes. The present study shows that environmental Mg^{2+} used in preparation does influence the responses of membranes to cationic agents. Variations in the amount of Mg^{2+} remaining bound to membranes prepared in low Mg^{2+}-shocking fluids could account for all of the fluctuations in the CTAB and β-lysin curves.

The data for influences of mixtures of protein and CTAB on ATPase proved to be as complex as is the dose response of this enzyme to CTAB [10]. A stimulation equal to the sum of the effects achieved with individual compounds present at their optimum concentrations suggests the existence of two sites for action. However, if the sites lie in close proximity, such a combination might not produce an additive effect due to steric or electrostatic interference. In the latter case, a mixture of one agent present at its optimum dose and the other agent at a low concentration may cause additive effects. To assume two closely situated sites, one should also demonstrate an additive effect with a mixture where the two agents are reversed in relative concentration. These criteria were fulfilled provided the level of detergent was at the low or high point of the second peak of CTAB stimulation, but not when detergent was present at a dose in the region of the first peak. The answer to this paradox must lie in the interrelationship of the two peaks in stimulation by CTAB [10]. The present findings might be expected if these peaks reflect two adjacent ATPase sites

rather than one, and if the site susceptible to 10—20 μg of CTAB per ml of the assay is also susceptible to stimulation by cationic proteins. It is of interest that the degrees of stimulations by cationic protein as well as by CTAB at 15 μg/ml compare favourably [10].

Regional anionic phospholipids are likely candidates for the target sites of cationic agents. The detergent effect of CTAB has been attributed to an ability to combine electrostatically with and to subtly rearrange phospholipids, thus producing channels in the hydrophobic barrier [14]. Stimulation of mitochondrial ATPase induced by arginine-rich histone [15] was reversible with anionic phospholipids as were the stimulation and inhibition influences of CTAB on the *B. subtilis* enzyme [10]. Concentrations of phospholipid in excess of that required to reverse either CTAB-induced inhibition or CTAB-induced stimulation caused secondary stimulation of enzyme activity [10]. The latter was due to the combined actions of CTAB and phospholipid since the effects of the phospholipid alone could not account for this phenomenon. These collective findings lead us to suggest a regulatory function for cations and phospholipids, a conclusion which has also been put forth by Lastras and Muñoz [16] concerning their observations on the properties of bound versus free ATPase of *Micrococcus lysodeikticus*.

Investigations on fluidity of membranes and membrane functions provide some clues as to the nature of such a regulatory mechanism operative on ATPase. In *Escherichia coli* [17] and *Mycoplasma laidlawii* [18] there is a requirement for fluidity of the membranes for membrane transport systems to operate. In higher animals the $(Na^+ + K^+)$-ATPase of kidney medulla is operative when the membrane is in the liquid-crystal state [19]. Grisham and Barnett [19] concluded that a phase transition affects enzyme activity by induction of a conformational change in the protein. Pertinent to the present work are results of studies using purified mono- and mixed-lipid bilayers [20]. Divalent cations, UO_2^{2+}, Ca^{2+}, and Mg^{2+} were shown to raise the gel to liquid crystalline transition temperature, while electrostatic binding of basic proteins or polypeptides to the anionic head groups of the phospholipids lowered the transition temperature by 3—7°C [20]. Residual Mg^{2+} in *B. subtilis* membranes might result in localized or regional shift of membrane lipids surrounding ATPase to the gel state, thus causing a lower overall observed control activity. Interaction of the cationic agents with the polar head groups of phospholipids would result in a localized gel to liquid transition and thus a stimulation of ATPase activity.

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