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EXPERIMENTAL ARTICLES

The Role of Microbial Dormancy Autoinducers in Metabolism Blockade

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Abstract—Alkyl-substituted hydroxybenzenes (AHBs), which are autoinducers of microbial dormancy (d_1 factors), were found to stabilize the structure of protein macromolecules and modify the catalytic activity of enzymes. In vitro experiments showed that C_6-AHB at concentrations from 10^{-4} to 10^{-2} M, at which it occurs in the medium as a true solution and a micellar colloid, respectively, nonspecifically inhibited the activity of chymotrypsin, RNase, invertase, and glucose oxidase. C_6-AHB-induced conformational alterations in protein macromolecules were due to the formation of complexes, as evidenced by differences in the fluorescence spectra of individual RNase and C_6-AHB and their mixtures and in the surface tension isotherms of C_6-AHB and trypsin solutions. Data on the involvement of dormancy autoinducers in the posttranslational modification of enzymes and their inhibition will provide further insight into the mechanisms of development and maintenance of dormant microbial forms.

Key words: dormancy, dormancy autoinducers, d_1 factors, alkyl-substituted hydroxybenzenes, metabolism blockade, structural modification of enzymes

There is increasing current interest in the mechanisms of microbial dormancy, a resting state characterized by the reversible inactivation of cellular metabolism associated with an increase in cell resistance to environmental stresses. The knowledge of the molecular mechanisms of dormancy can provide further insight into the general principles of regulation of cell metabolism and cytodifferentiation and into the strategy of microbial survival and adaptation to varying environmental conditions.

Investigations of resting microbial forms (bacillar endospores, bacterial cysts, and fungal spores) have led to the following three independent hypotheses explaining the development and maintenance of the dormant state in microorganisms:

(1) production of metabolic autoinhibitors (e.g., spore germination inhibitors), whose release from spores results in the cessation of the dormant state [1];

(2) dehydration of the spore protoplast due to changes in the ultrastructure of cells, the phase state of membranes, and their selective permeability [2, 3];

(3) reversible inactivation of enzymes due to the formation of thermostable complexes with dipicolinate calcium [4].

It should be noted that there is some controversy between experimental data and the above hypotheses, probably accounted for by the great diversity of the structural, chemical, and biochemical properties of resting microbial forms. At the same time, the general properties of resting forms suggest that the mechanisms of their formation must be similar. From this point of view, of much interest is the role of low-molecular-weight regulatory substances involved in the formation and maintenance of metabolically dormant microbial forms.

Earlier, we described alkyl-substituted hydroxybenzenes (AHBs) as autoregulatory d_1 factors inducing the conversion of vegetative microbial cells to dormant cystlike refractory forms [5–10]. Factors d_1 modify the structure of membranes by increasing the microviscosity of the lipid stroma due to hydrogen bonding between the hydroxyl groups of the aromatic ring of the factors and membrane lipids. As a result, d_1 factors enhance the permeability of membranes to monovalent ions, induce the dehydration of the cell protoplast, affect the activity of membrane-associated enzymes,

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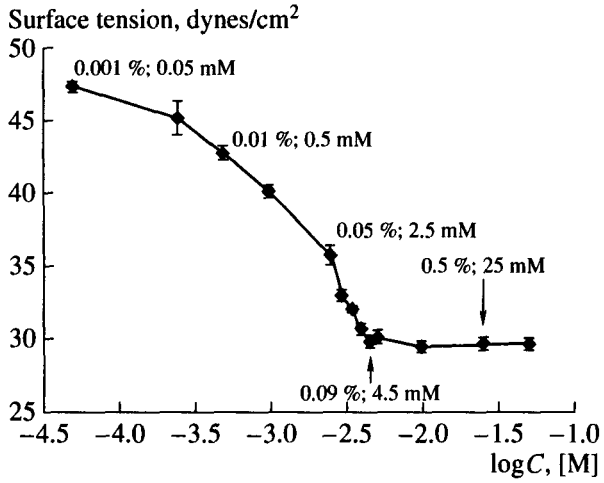

Fig. 1. Concentration dependence of the surface tension of the water–ethanol solutions of C_6-AHB. The arrow indicates the CMC of C_6-AHB.

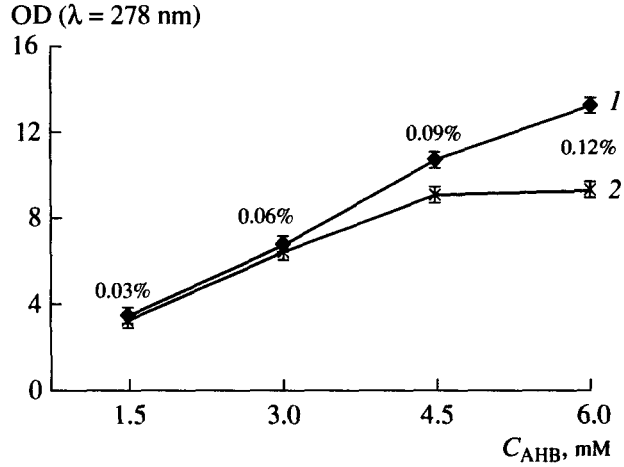

Fig. 2. Concentration dependence of the optical density of the water–ethanol solutions of C_6-AHB (*1*) before and (*2*) after ultracentrifugation at 180000 g.

and exert antioxidant effects [11–14]. All this cannot, however, explain the inhibition of various cytosolic depolymerases responsible for cell autolysis in the absence of energy-generating processes [15]. In our opinion, the inhibition of biosynthetic and autolytic processes in the dormant state may be due to conformational alterations in the enzymes located in various cell compartments.

The aim of the present work was to study the modifying effect of d_1 factors on protein macromolecules leading to a nonspecific inhibition of enzymes and a metabolism blockade in dormant cells.

MATERIALS AND METHODS

Experiments were performed with the extracellular alkaline RNase of *Bacillus intermedius* (EC 3.1.27.5) purchased from the Institute of Organic Synthesis, Academy of Sciences of Latvia (the activity of this RNase was 1 million units/ml); chymotrypsin (EC 3.4.21.1) from Merck (Germany); invertase (EC 3.2.1.26) from Sigma (United States), and glucose oxidase (EC 1.1.3.4) from Sigma.

RNase activity was determined from the rate of accumulation of acid-soluble products of RNA hydrolysis [16]; chymotrypsin activity, from the rate of hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) [17]; invertase activity, from the rate of reducing sugar release from sucrose assessed with 3,5-dinitrosalicylic acid [18]; and glucose oxidase activity, from the rate of glucose consumption assessed with potassium ferricyanide [19].

Alkyl-substituted C_6-hydroxybenzene (C_6-AHB), an amphiphilic compound with M = 196 and pK_a = 9, was used as a d_1 factor. C_6-AHB was added to reaction mixtures in the form of an ethanol solution to give the final concentration of ethanol within 3–5 vol %. Enzymes and substrates were preincubated with a d_1 factor at 25°C for 15–30 min. Blank reaction mixtures contained equivalent amounts of ethanol.

The critical micelle concentration (CMC) of C_6-AHB in aqueous solutions was determined either from the position of a kink point on the surface tension isotherm of solutions derived using a Wilhelmy plate [20] or by measuring the extinction of C_6-AHB at λ_{max} = 278 nm (Specord spectrophotometer; 10-mm cuvette) in the supernatants obtained by the centrifugation of the d_1 factor solutions at 180000 g. The micelle size was determined by measuring the light scattering of colloids using a setup designed at the Laboratory of Interphase Phenomena of the Nesmeyanov Institute of Organoelement Compounds and a 600-nm laser.

The excitation and emission spectra of solutions of RNase, the d_1 factor, and their mixtures were measured using an automatic Signa-4M spectrofluorimeter (Latvia).

The data presented are the means of five replicated measurements performed in three independent sets of experiments. Data were statistically processed by calculating standard deviations and using Student's *t*-test with significance level $P < 0.05$.

RESULTS AND DISCUSSION

To know the state of the d_1 factor in the medium (true solution or micellar colloid), it was necessary to determine the critical micelle concentration (CMC) of C_6-AHB in aqueous solutions. As can be seen from Figs. 1 and 2, in a 5% solution of ethanol in water, C_6-AHB forms a true solution at concentrations lower than 0.09% (4.5 mM) (the solution is stable at concentrations not exceeding 0.05% (2.5 mM)) and a micellar colloid system at concentrations higher than 0.09%. Thus, the CMC of this d_1 factor is 0.09%. This concentration of C_6-AHB corresponds to the kink point on the surface tension isotherm (Fig. 1) and to the statistically

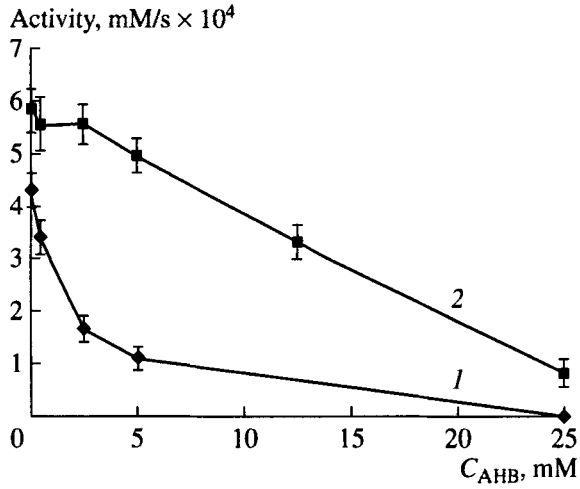

Fig. 3. Effect of C_6-AHB on the activity of (*1*) glucose oxidase and (2) invertase.

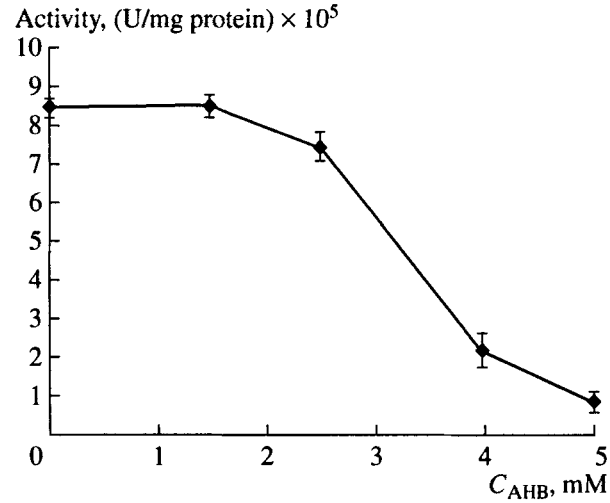

Fig. 4. Effect of C_6-AHB on the activity of *B. intermedius* RNase.

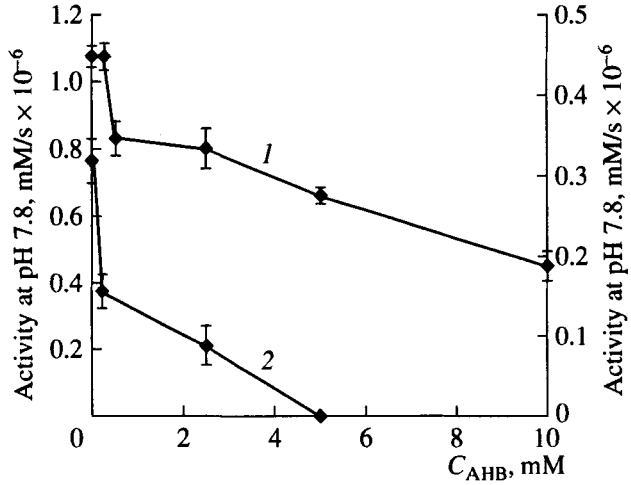

Fig. 5. Effect of C_6-AHB on the activity of chymotrypsin at (*1*) pH 7.8 and (2) pH 6.0.

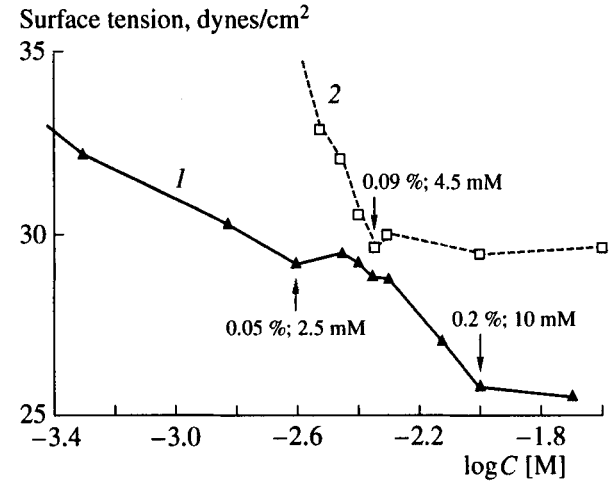

Fig. 6. Effect of the C_6-AHB content on the surface tension of the water–ethanol solutions of (*1*) a C_6-AHB–trypsin (2.5 mg/ml) mixture and (2) individual C_6-AHB.

significant difference between the extinctions (and, therefore, the concentrations) of C_6-AHB in 5% ethanol before and after centrifugation at 180000 g (Fig. 2). At concentrations of 0.1% (5 mM) and higher, C_6-AHB forms stable micelles about 100 μm in diameter. It should be noted that the shape of the surface tension isotherm of C_6-AHB (Fig. 1) is typical of surface-active substances; this fact is important for understanding the mechanism of action of this d_1 factor as a dormancy autoinducer.

According to our hypothesis, the mechanism of action of the d_1 factor during the development and maintenance of dormant states involves, among other effects, a nonspecific inhibition of enzymes (Figs. 3–5). The concentrations of C_6-AHB inducing 50% inhibition were different for different enzymes: 0.07% (3.5 mM) for RNase, 0.2% (10 mM) for chymotrypsin, 0.05% (2.5 mM) for glucose oxidase, and 0.3% (15 mM) for invertase. At the CMC value, C_6-AHB suppressed the activities of RNase, chymotrypsin, glucose oxidase, and invertase by 90, 34, 57, and 28%, respectively. Therefore, C_6-AHB inhibited enzymes irrespective of its state in the medium (true solution or micelles).

The mechanism of action of C_6-AHB at concentrations below CMC can be explained in terms of its ability to form hydrogen bonds (due to the hydroxyl groups of its aromatic ring) and hydrophobic bonds (due to hydrophobic alkyl substituents) with protein macromolecules. At concentrations higher than the CMC value, the d_1 factor probably inhibits enzymes by forming stable micellar systems, which serve as immobiliza-

tion matrices. This assumption agrees with the data reported by other authors, who showed that the alkylresorcinols of azotobacters can be components of membrane lipids [21] and that the association (or dissociation) of enzymes with hydrophobic lipid compounds organized in a micellar membrane-like phase is accompanied by the structural rearrangement of enzyme molecules. The rearrangement involves, in particular, changes in the proportion of α-helices [22], which alters the kinetic properties of enzymes [23]. All these effects of the nonspecific inhibition of enzymes by AHBs may take place in vivo during the development of the dormant state of resting microbial forms, when the molecular states of AHB are different in different cell compartments.

It should be noted that enzyme activities were assayed in vitro at optimal pH values (8.5 for RNase, 7.8 for chymotrypsin, 6.5 for glucose oxidase, and 4.5 for invertase), whereas pH values in different cell compartments are different and, moreover, depend on the physiological state of cells; this may essentially influence the activity of cellular autoregulators. The dependence of the biological activity of d_1 factors on pH has been shown with respect to the induction of cystlike dormant microbial forms and the inhibition of the respiration of intact cells and membrane preparations [8, 13]. The activity of d_1 factors was higher at alkaline pH values, when their molecules are protonated and, therefore, are more reactive to form hydrogen and hydrophobic bonds.

The pH dependence of the inhibitory action of C_6-AHB was studied in experiments with chymotrypsin (Fig. 5). At pH 6.0 and 7.8, a 50% inhibition of chymotrypsin activity by C_6-AHB was observed when it was used at concentrations of 0.005% (0.25 mM) and 0.2% (10 mM), respectively. At acidic pH values, 0.1% (5 mM) C_6-AHB completely inactivated chymotrypsin.

These results may be explained through conformational changes in enzyme molecules induced by d_1 factors. Such an explanation was confirmed by two independent methods.

The first method demonstrated the ability of the d_1 factor to form complexes with biomacromolecules, as evidenced by measurements of the surface tensions of solutions of individual C_6-AHB and its mixtures with trypsin (Fig. 6). In contrast to the case of individual C_6-AHB (curve 2), the kink point on the surface tension isotherm of C_6-AHB–trypsin mixtures (curve *1*), which corresponds to CMC, is displaced toward higher concentrations of C_6AHB; this is evidently due to the formation of complexes between trypsin and C_6-AHB. The surface tension of solutions containing 0.01–50 mg trypsin/ml ranged from 54 to 40 dynes/cm^2.

The complicated shape of the surface tension isotherm of mixtures is due to several factors: protein–AHB interactions at AHB concentrations lower than 0.05% (2.5 mM), changes in the orientation of AHB molecules on the surface of protein molecules at AHB

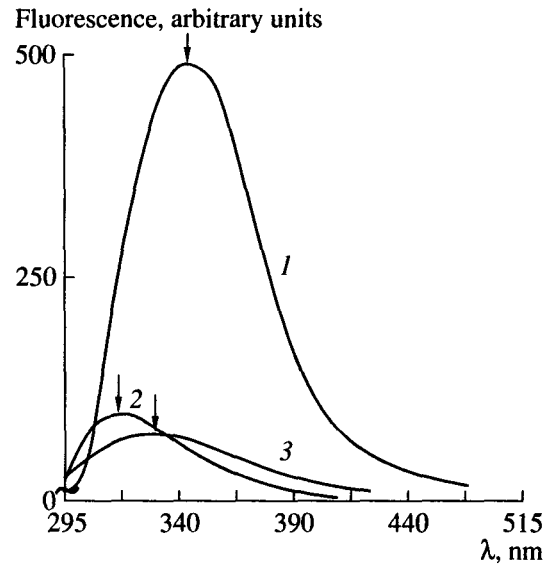

Fig. 7. Fluorescence spectra of (*1*) RNase (λ_{ex} = 278 nm; λ_{em} = 341 nm), (*2*) C_6-AHB (λ_{ex} = 269 nm; λ_{em} = 312 nm), and (*3*) mixture of 0.05 μM RNase and 0.1 mM C_6-AHB.

concentrations between 0.05 and 0.1%, and the enlargement of protein–AHB complexes with the increase of the AHB concentration to 0.2%. The enlargement of complexes manifested itself in the opalescence of solutions and in a decrease in their surface tension. All this suggests that enzymes and AHBs interact due to the formation of hydrogen and hydrophobic bonds, rather than due to the immobilization of enzymes on the AHB matrix. Such immobilization is, however, possible on the Ca^{2+}–AHB matrix resembling the supramolecular Ca^{2+}–dipicolinic acid complexes of endospores [4].

The second method is based on the analysis of the fluorescence characteristics of RNase, AHB, and their mixtures. The excitation and emission spectra of RNase are characterized by λ_{ex} = 278 nm and λ_{em} = 341 nm. The respective values for C_6-AHB are λ_{ex} = 269 nm and λ_{em} = 312 nm (Fig. 7).

The fluorescence characteristics of RNase–AHB mixtures depended on the molar proportion of the components. At a molar ratio of 20 (in this case, the mixture contained 0.1 mM C_6-AHB and 5 μM (0.1 mg/ml) RNase), the emission spectrum of the mixture at λ_{ex} = 274 nm corresponded to that of individual RNase with λ_{em} = 341 nm, although the intensity of fluorescence was lower than in the case of individual RNase. At a molar ratio of 2000 (1 mM AHB and 0.5 μM RNase), the emission spectrum of the mixture at λ_{ex} = 270 nm corresponded to that of individual C_6-AHB with λ_{ex} = 312 nm. At a medium molar ratio of 200 (0.1 mM C_6-AHB and 0.5 μM RNase), both the intensity and the wavelength of the fluorescence maximum (λ_{em} = 279 nm and λ_{ex} = 328 nm) differed from those of the individual components (Fig. 7). In spite of some difficulties in the interpretation of these data, it can be suggested that

physicochemical interactions between RNase and AHB lead to the formation of a weakly fluorescent RNase–AHB complex and the inhibition of enzyme activity. This suggestion is consistent with the results obtained by the first method.

To conclude, we showed that alkyl-substituted hydroxybenzenes, the autoregulatory d_1 factors of microorganisms inducing their transition to the dormant state, are the natural structural modifiers of enzymes and the lipid stroma of membranes. Noncovalent interactions between AHBs and enzymes give rise to AHB–protein complexes with low or zero enzymatic activity. The properties of such complexes depend on the physicochemical parameters of the medium and on the hydrophobicity of AHB molecules, whose degree differs in different microbial species and depends on their growth phase. As was shown in experiments with *Pseudomonas carboxydoflava* [5–7], *Methylococcus capsulatus* [8], *Bacilluis cereus* [5], *Saccharomyces cerevisiae* [9], and other microorganisms, the effect of autoregulatory d_1 factors on microbial cells is implemented via the modification of the structure and functional activity of membranes [11–13]. Further studies of the d_1 factor–mediated post-translational autoregulation of enzymes can provide more insight into the mechanisms of microbial dormancy and senescence, as well as of cell response to stresses.

Thus, the polyfunctional AHBs (d_1 factors) act as inducers of the cell protoplast dehydration [11, 12] and structural modifiers of membranes and enzymes, thereby blocking metabolism in dormant microbial forms. Furthermore, they are inhibitors of spore germination, which is the subject of our next publication.

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