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REGULATORY RESPONSES OF ARGININE DEIMINASE IN WHOLE CELLS OF *CLOSTRIDIUM SPOROGENES*

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Summary

Arginine deiminase (EC 3.5.3.6) has been shown to have regulatory properties. The activity was observed to be sigmoidal with respect to substrate concentrations. Addition of histidine to the system caused the abolition of sigmoidal responses. The regulatory properties of the enzyme as well as the desensitising action of histidine could also be demonstrated with whole cell suspensions. The pH of the system also seemed to influence modulations in the enzyme.

Introduction

Allosteric regulation of enzyme activity is being investigated essentially with reference to subunit structure and conformational alterations in presence of the effector molecules using purified enzyme preparations [1—4]. It is not clear, however, if the kinetic parameters adopted for establishing the cooperative interactions in vitro would hold true for the enzymes participating in the integrated metabolic sequences in the intact cells. The possible significance of allosteric behaviour of enzymes in vivo has been stated in the reviews of Kirschner [5] and Atkinson [6]. Recently, Kuhn et al. [7] reported kinetic studies on purified phosphofructokinase employing near cellular conditions and Bagnara et al. [8] studied the properties of amidophosphoribosyltransferase in intact Ehrlich ascites tumour cells. Attempts have also been made by Serrano et al. [9] and Reitzer and Neet [10] to investigate the regulatory behaviour of allosteric enzymes in situ using yeast cells rendered permeable to substrate and effector molecules. The present paper describes the regulatory nature of arginine deiminase (EC 3.5.3.6) in *Clostridium sporogenes* both in cell free preparations as well as in intact cells. A preliminary report of this observation has been communicated earlier [11].

Materials and Methods

L-Histidine-HCl, DL-citrulline, 2,5-diphenyloxazol (PPO) and 2,2^1-*p*-phenylen-bis (5-phenyloxazol) (POPOP) were purchased from Sigma Chemical Co., USA. L-Arginine-HCl was obtained from V.P. Chest Institute, Delhi, India and diacetyl monoxime from E. Merck, Germany. L-[U-^{14}C] Arginine (30 Ci/mol) was the product of the Isotope Division, of the Bhabha Atomic Research Centre.

Growth and harvesting of cells

Clostridium sporogenes (ATCC 19404) was routinely cultured and stored in a cooked meat medium (Difco).

The organism was grown in a medium composed of tryptone 3%, ammonium sulfate 1%, yeast extract 0.1% and sodium thioglycollate 0.1% (12). 16 h old vegetative cells were inoculated in a sterile medium of the same composition at 20% level and incubated further at 37° C for a period of 4 h. At the end of incubation period, the exponentially growing cells were harvested, washed once with 0.05 M Tris · HCl buffer (pH 6—9) as required in individual experiments and the pellet was resuspended in the same buffer. Cell suspensions, adjusted to an absorbance of 1.0 at 600 nm on a Bausch and Lomb Spectronic 20 Spectrocolorimeter, were used for enzyme studies in whole cells. At this cell density, the protein content of the cell suspension was 6—7 mg/ml.

Preparation of cell free extracts

For the preparation of cell free extracts, cells grown as described above were suspended in 0.05 M Tris · HCl buffer at an approximate cell concentration of 1.0 mg dry weight per ml and sonicated at ice temperature for a period of 4 min in a MSE 60 W Ultrasonic cell disintegrator. The extract was centrifuged at 3000 × *g* for 20 min at 0° C to remove the unruptured cells and cell debris and the supernatant was used for the enzyme assay.

Assay of arginine deiminase

Arginine deiminase activity was determined in whole cells as well as cell free preparations of *Cl. sporogenes*, using arginine · HCl as the substrate. Citrulline liberated in the course of the reaction was estimated by the method of Oginsky [13].

The assay system consisted of 0.45 ml of whole cell suspension or the cell extract preincubated at 37° C for 10 min, in 0.05 M Tris · HCl buffer adjusted to the required pH values, arginine · HCl (final concentration 0.1—100 mM) and 0.05 M Tris · HCl buffer in a total volume of 1 ml. The assay mixture was incubated for 1 h at 37° C. The reaction was terminated by the addition of 1 ml 10% trichloroacetic acid and the citrulline liberated was estimated [13].

Estimation of protein

Protein content in whole cells and in cell free extracts was determined by digesting the aliquots with 2.5 M H_2SO_4 containing 150 mg sodium selenite per liter. The nitrogen of the digested samples was estimated by Nesslerisation [14] and protein was calculated by multiplying the nitrogen values by 6.25.

Radioactivity experiments

An incubation mixture (1 ml) consisting of 0.45 ml cell suspension prepared as mentioned earlier and 100 mM L-arginine · HCl containing 5 μCi L-[U-^{14}C]arginine (specific activity 30 Ci/mol) was held at 37°C for 5 min. Aliquots (0.2 ml) were withdrawn at intervals of 30 s and pipetted on Whatman No. 3 filter discs in a millipore filter assembly and immediately washed with 7 ml Tris · HCl buffer pH 7.5. The discs were dried and counted for radioactivity using a Beckman Liquid Scintillation Spectrometer Model LS-100 in presence of 3 ml scintillation fluid consisting of 0.05 g POPOP and 15.0 g PPO per liter toluene [15].

Results

Kinetics of arginine deiminase in whole cell assay system

Since the present experiments are carried out with whole cells of *Cl. sporogenes* for assaying arginine deiminase activity, some kinetic parameters had to be established. Data given in Fig. 1a and b show that the enzyme activity was linear with time of incubation as well as cell concentration.

The kinetics of uptake of L-[U-^{14}C]arginine by whole cells is presented in Fig. 2. It was observed that the uptake of arginine was rapid following a normal hyperbolic pattern with respect to time. These observations clearly indicate that the permeability of arginine was not a limiting factor in studying arginine deiminase in whole cells of *Cl. sporogenes*.

The pH activity curves of arginine deiminase (Fig. 3) pointed to two pH optima of 6 and 6.5 at 10 mM and 60 mM arginine respectively, suggesting the possible existence of the enzyme in at least two molecular conformations at these substrate concentrations. These changes might also reflect different degrees of saturation and hence variation in binding and catalysis.

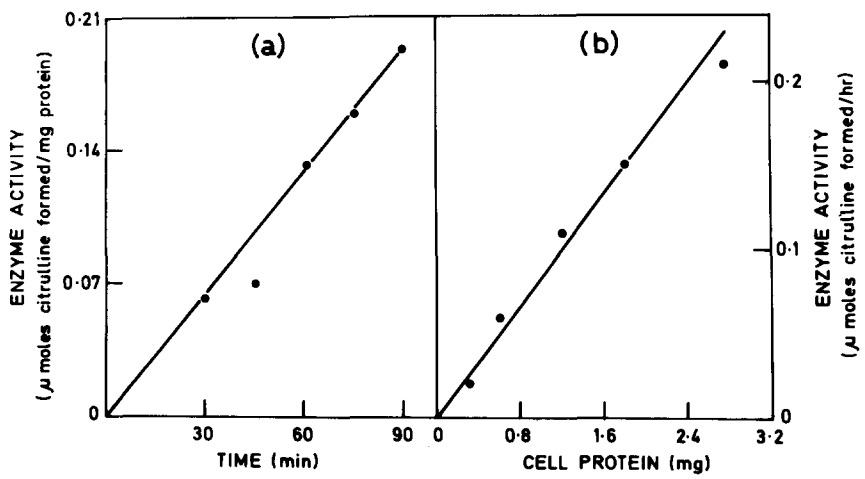

Fig. 1. Arginine deiminase activity in whole cells of *Cl. sporogenes* as a function of (a) incubation time and (b) cell protein concentration. Details of other experimental conditions are stated in the text. The concentration of arginine used in the assay system was 10 mM.

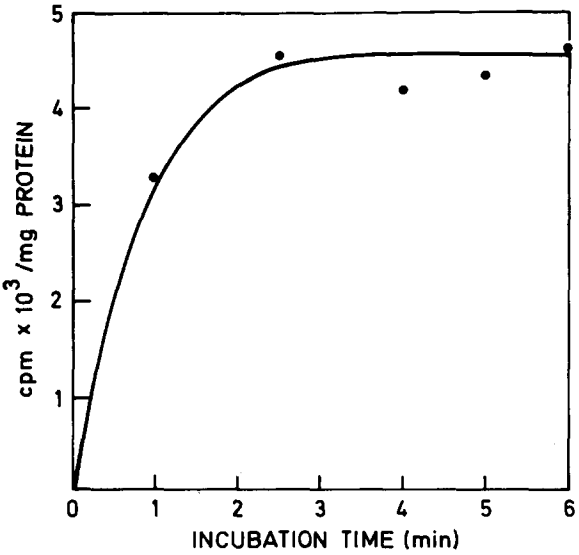

Fig. 2. Uptake of L-[U-^{14}C]arginine by cells of *Cl. sporogenes*.

Activation of arginine deiminase by histidine

Histidine was found to activate the enzyme at pH 7.0 and the maximum activation of the enzyme was attained at 30 mM concentration of histidine (Fig. 4). It was observed that only histidine was able to cause this activation of arginine deiminase, while other basic amino acids like lysine and ornithine were ineffective (Table I). Among the metabolites of histidine including histamine,

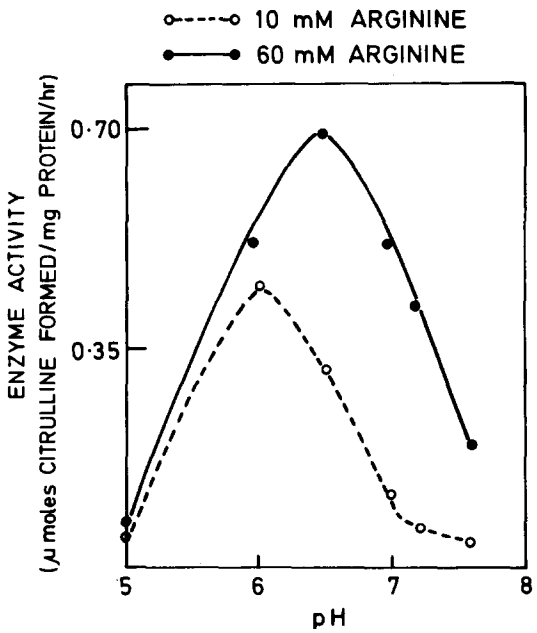

Fig. 3. Effect of pH on arginine deiminase activity in whole cells of *Cl. sporogenes*.

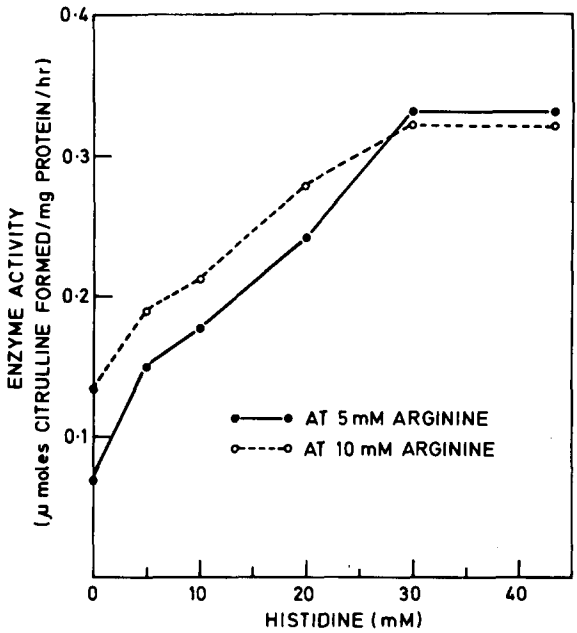

Fig. 4. Effect of varying concentrations of histidine on arginine deiminase activity in whole cells of *Cl. sporogenes*. The cells equivalent to 3 mg of protein were suspended in 0.45 ml Tris · HCl buffer, pH 7.0, and were incubated at 37°C for 10 min with histidine (pH of aqueous solution adjusted to 7.0 with 2 M NaOH) at given concentrations before the addition of arginine.

urocanic acid and imidazole, only histamine was partially effective in activating the enzyme. The incorporation of actinomycin-D (10 μg) or chloramphenicol (100 μg) in the assay system did not influence the activating effect of histidine, suggesting that the enhancement in activity was not due to de novo synthesis of the enzyme (Table I).

Histidine dependent activation of arginine deiminase was examined at pH values ranging from 5.0 to 9.0 (Fig. 5). It was observed that no significant

TABLE I

EFFECT OF VARIOUS ADDITIONS IN THE ASSAY SYSTEM ON THE ACTIVITY OF ARGININE DEIMINASE IN WHOLE CELLS OF *CL. SPOROGENES*

Enzyme activity is expressed as μmol citrulline formed/mg protein/h. The complete system has been described in the text. The above additions were made to the complete system, at the concentrations indicated. Arginine concentration used in the enzyme assay was 10 mM.

Additions to the complete system	Enzyme activity
None	0.14
Histidine (10 mM)	0.27
Lysine (10 mM)	0.14
Ornithine (10 mM)	0.14
Imidazole (10 mM)	0.14
Histamine (10 mM)	0.19
Urocanic acid (10 mM)	0.15
Histidine (10 mM) + actinomycin D (10 μg)	0.27
Histidine (10 mM) + chloramphenicol (100 μg)	0.27

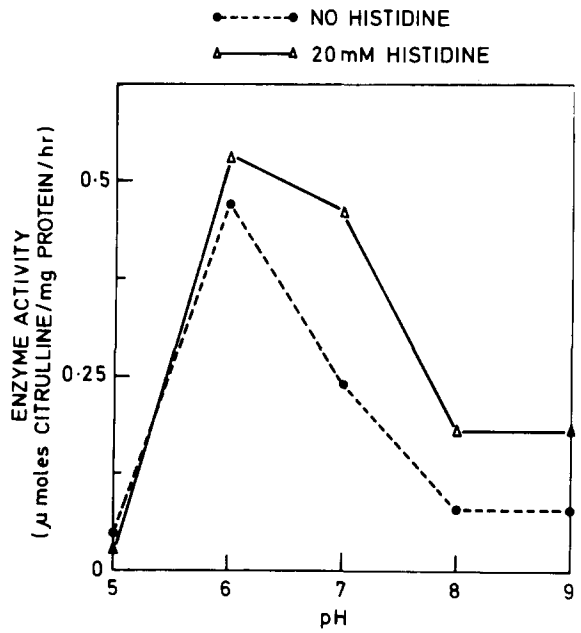

Fig. 5. The influence of pH on the activation of arginine deiminase in whole cells of *Cl. sporogenes* by histidine. Concentration of the substrate in the assay was 10 mM.

activation of the enzyme occurred at pH 6.0. However, at pH 7.0 and above the enzyme showed marked enhancement in activity.

Effect of substrate concentration on the enzyme activity

Arginine deiminase activity in whole cells was observed to be sigmoidal with respect to substrate concentration at pH 7.0 (Fig. 6). The addition of histidine to the assay system at pH 7.0 altered the sidmoidal nature of the

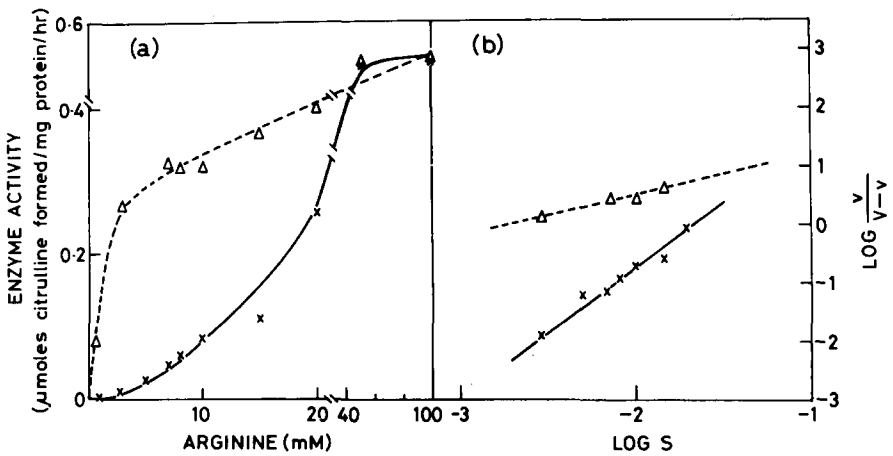

Fig. 6. Effect of substrate concentration on arginine deiminase activity in whole cells of *Cl. sporogenes* in presence and absence of histidine. This is shown in (a) while Hill plots are presented in (b). X------X, no histidine; △------△, 40 mM histidine.

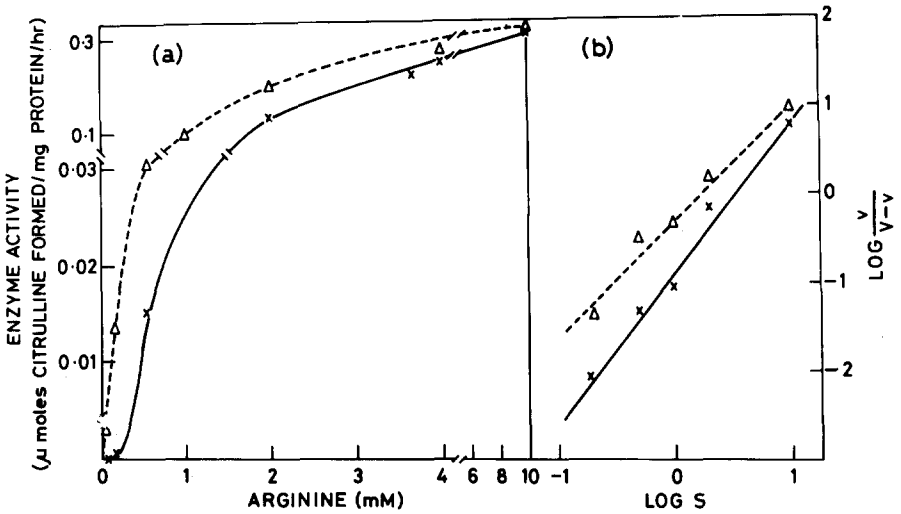

Fig. 7. Effect of substrate concentration on arginine deiminase activity in cell free extract of *Cl. sporogenes*. (a) enzyme activity versus substrate plot (b) Hill plot. X———X, no histidine; △- - - - -△, 20 mM histidine.

activity curve. It was observed that in presence of 40 mM histidine the enzyme obeyed normal Michaelis-Menten kinetics. The analysis of this data by Hill plot showed that the binding coefficient for arginine was 2.0 in the absence of histidine while it was reduced to 0.8 when 40 mM histidine was added to the system.

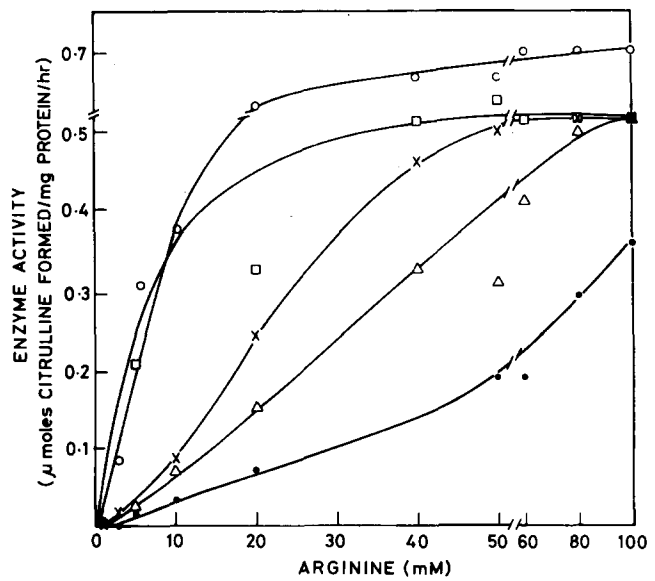

Fig. 8. Effect of pH on the activity of arginine deiminase in whole cells of *Cl. sporogenes* at varying substrate concentrations. The reaction mixture contained cells equivalent to 3 mg of protein in Tris—HCl buffer adjusted to pH ranging from 6.0 to 7.6 and arginine in a total volume of 1.0 ml. □———□, pH 6.0; ○———○, pH 6.5; X———X, pH 7.0; △———△, pH 7.2 and ●———●, pH 7.6.

The cooperative interaction of the enzyme with arginine could also be seen with cell free extracts as evidenced by the sigmoidal activity curve (Fig. 7a). However, the extent of modulation appeared to be less pronounced. The addition of histidine (20 mM) completely reversed the sigmoidal behaviour. The Hill coefficients for the enzyme in the absence and presence of histidine were 1.7 and 1.3, respectively (Fig. 7b).

The profound influence of pH on the activity of arginine deiminase was indicated by a shift in pH optimum of the enzyme at varying substrate concentrations (Fig. 3). It was also apparent that the sigmoidal responses were obtained at pH 7.0 and above as shown in Fig. 8. At lower pH values the activity of the enzyme was hyperbolic with reference to substrate concentration. The Hill plots made from this data showed that the binding coefficients were increasing from 0.94 to 2.4 with the rise in pH from 6.0 to 7.6 indicating enhanced degree of interactions at higher pH values.

Discussion

Arginine deiminase is the first enzyme of arginine dihydrolase system generating ATP through utilisation of arginine in some microorganisms [16,17]. The presence of this enzyme has been reported in *Streptococcus lactis* [18], *Pseudomonas fluorescens* [19], *Clostridium perfringens* [20], *Clostridium botulinum* [21] and yeast [22]. This enzyme from *S. faecalis* and from some strains of *Mycoplasma* has also been characterised [17,23]. The present studies however, show that arginine deiminase from *C. sporogenes* has regulatory properties. The sigmoidal response with the substrate and the activation by histidine, are not only observed with cell free extracts, but also with whole cells. This, therefore, seems to be one of the rare instances where modulation of enzyme activity in vivo has been observed. In order to demonstrate the allosteric behaviour of enzymes in intact cells, it is necessary to consider factors such as age of cells and their commitment to differentiation. It has been observed in our laboratory that exponentially growing cells were most suitable for studying arginine deiminase activity. However, during sporulation, the activity of this enzyme could not be detected with whole cell suspension (Venugopal, V. and Kumta, U.S., unpublished). This could presumably be correlated with changes in permeability, since the activity was apparent only when cells were disrupted by sonication. In the present experiments, permeability of arginine did not appear to be a limiting factor since the uptake of [U-^{14}C] arginine by the cells showed a hyperbolic relationship. The uptake of labelled arginine was also not affected by the addition of histidine to the system.

The validity of the whole cell enzyme assay has been evidenced by the linear relationship of the activity with time and cell concentration. The responses observed with the cell free extract and the whole cell respectively also showed similarities. The experiments using actinomycin-D and chloramphenicol clearly indicated that histidine induced activation of arginine deiminase was not due to de novo synthesis of the enzyme.

The sigmoidal responses of the enzyme with varying concentrations of the substrate were not seen in the presence of histidine in the system. This is in

keeping with the characteristics reported for a positive modifier of allosteric enzyme [1,19].

The allosteric function of enzymes has been reported to be influenced by pH [19,24,25]. The loss of sigmoidal behaviour at acidic pH and nonlinear reciprocal plot at pH 7.0 may suggest conformational changes in the present enzyme. The lack of activation by histidine at pH below 7.0 might indicate similar mechanisms for the activation of the enzyme by histidine as well as by low pH. The reduction of Hill coefficient from two to unity obtained by the addition of histidine and that observed at acidic pH may also substantiate the observations with respect to conformational changes.

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