Periplasmically located α -santonin binding factor in *Sphingomonas paucimobilis* strain S ATCC 43388

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Abstract A marked reduction in uptake of α -santonin, accompanied by loss of ability of cells to transform the substrate, is observed on shocking *Sphingomonas paucimobilis* strain S ATCC 43388 cells by freeze – thaw method. The shock fluid shows a 26% quench in fluorescence at 350nm on incubation with the substrate. Addition of shock fluid to the freeze thawed cells restores both uptake as well as transformation of α -santonin to near normal.

Keywords Periplasmic proteins · Shock fluid · Fluorescence · *Sphingomonas*

Introduction

Several sesquiterpene lactones have received considerable attention in pharmacological research due to their potent anti-neoplastic, anti-inflammatory activities. The sesquiterpene lactones have also been reported to exert cytostatic and cytotoxic effects against tumour cells. α -Santonin, a sesquiterpene lactone, is commonly found in plants belonging to Compositae family. α -Santonin is known to be a potent anti-parasitic agent, and in its α,β forms exhibits anti-pyretic activity in a fashion similar to that of dopamine¹. It is known to be effective against several species of fungi via the inhibition of spore germination² and hence is used as biocontrol agent. Chemically modified derivatives of α -santonin are also known to confer the ability to induce the differentiation of human promyelocytic leukemia cells³. As it had attained significance as potential parent compound several workers have used microorganisms such as *S. aureofaciens*, *S. cineriacatus* to transform α -santonin and obtained lumisantonin and 1,2 dihydrosantonin as the products^{4,5,6}.

Bacterial strain S ATCC 43388⁷ identified subsequently as *Sphingomonas paucimobilis*⁸ utilizes crystalline, hydrophobic sesquiterpene α -santonin as a sole source of carbon, forming various intermediary products^{7,9,10,11,12} by two independent pathways. During the study on α -santonin degradation pathway in our laboratory ten intermediate products, six in native form and four in the form of 2,4 DNP derivatives were isolated and characterised^{9,10,11,12}. The products were isolated using metabolic inhibitors like DCCD and Semicarbazide. α -santonin is metabolised via dihydrosantonin as major energy yielding pathway but in presence of semicarbazide it is utilised via dihydroxysantonin. The enzyme α -santonin 1,2 reductase catalyses the reduction of

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α -santonin to dihydrosantonin the first step in α -santonin degradation⁹. Dihydrosantonin undergoes hydroxylation to give 4,5 dihydroxy products that are subsequently utilised.

Simultaneously, a study was carried out in our laboratory on transport of α -santonin in *S. paucimobilis* cells¹³. This study was intriguing as α -santonin is sparingly soluble, the dissolution of which is 166 $\mu\text{g/ml}$ and forms shiny white crystals in liquid medium and solid medium. Studies to test the presence of solubilising factor suggested that the utilisation is purely by a physical phenomenon and transport of α -santonin in *S. paucimobilis* cells does not require any external biological factor¹³.

The loss of periplasmic proteins on subjecting the Gram negative bacterial cells to osmotic shock or freeze thawing results in concomitant transport of hydrophilic substrates such as sugars, amino acids and inorganic ions^{14,15,16}. The study of hydrocarbon uptake is still in infancy, and detailed research is needed to thoroughly outline the process involved. The present work was aimed at studying the mechanism underlying α -santonin transport by *S. paucimobilis* cells.

We report herein the role of periplasmically located binding factor in the transport of α -santonin.

Materials and Methods

Organism: *S. paucimobilis* strain S ATCC 43388 was maintained on mineral medium agar containing 0.4% α -santonin at RT⁷.

Extraction of Periplasmic proteins: *S. paucimobilis* strain S cells release periplasmic proteins in the shock fluid, on subjecting to freeze-thaw cycles, osmotic shock and by chloroform; freeze thawing being the most effective method¹⁶. Periplasmic proteins were extracted routinely by two cycles of freezing (-20°C) and thawing of cells, which were previously adjusted to an absorbance of 4.5 at 450 nm^{16,17}. The supernatant shock fluid, passed through 0.45 μ millipore filter was analysed for protein content¹⁸ and stored at -20°C till further use. Thawed cells, devoid of shock fluid were suspended in 0.05 M phosphate buffer to an absorbance of 4.5 at 450 nm and were checked for α -santonin transport¹³ and transformation activity^{9,10,11,12}.

α -santonin binding assay: α -santonin binding activity in the shock fluid was measured by equilibrium dialysis assay¹⁶ and by spectrofluorometric analysis¹⁹. The excitation and emission scan of the ammonium sulphate-precipitated shock fluid, containing 500 μg proteins, with (0.2 μM) or without α -santonin was monitored on a Hitachi F-2000 fluorescence spectrophotometer.

Electrophoresis: Proteins were separated by discontinuous SDS PAGE²⁰ using 4% acrylamide stacking gel and 10% (w/v) acrylamide resolving gel.

Results and Discussion

Release of Periplasmic Proteins

Substrate binding proteins situated in periplasmic space play an important role in nutrient transport, demonstrated by their specific binding to the substrates and lack of transport of substrate in the cells devoid of such transport proteins²¹. Efficient release of periplasmic proteins from the organisms is entirely desirable. Several techniques have been developed which selectively and quantitatively release periplasmic proteins. Frequently used techniques are osmotic shocking (COSM), Freeze thawing and chloroform (CHLM) methods. However in our present work with *S. paucimobilis* strain S, FTM was more efficient and released more periplasmic proteins than COSM and CHLM¹⁶. This method also has selective advantages over other conventional methods, as it does not involve any chemical treatment such as lysozyme, EDTA, Chloroform or toluene and releases selectively periplasmic proteins without loss of apparent cell damage¹⁶.

SDS PAGE Profile and Densitograms of whole cell proteins of α -santonin grown cells subjected to repeated freezing and thawing show the diminished band at 46 kDa (Fig. 1B; Fig. 2-lane 2). Apparently, the same protein is preferentially released into the shock fluid (Fig. 1C; Fig. 2-lane 3), which contains about 100 μg total protein per ml, and shows, besides the major 46 kDa protein, several minor bands.

α -santonin binding activity of shock fluid

Shock fluid released by freeze thawing of *S. paucimobilis* cells shows an affinity for α -santonin. When dialyzed against saturated α -santonin solution, it binds 158 μg of α -santonin per ml as against only 98 μg in corresponding buffer control. Shock fluid extracted from cells with protein content of 150 $\mu\text{g ml}^{-1}$ binds α -santonin to super saturated level, 198 $\mu\text{g ml}^{-1}$, indicating that with increase in protein content, there is a proportional increase in the α -santonin binding capacity of the shock fluid. The disadvantages of sparing solubility of α -santonin in water (166 $\mu\text{g ml}^{-1}$) and insensitive colorimetric assay method could be overcome by fluorescence spectroscopic assay, a highly sensitive tool for monitoring protein ligand interactions. The shock fluid has an excitation maximum at 300 nm and emission at 350 nm as indicated in Fig. 3 (A); substrate α -santonin does not show any fluorescence at these wavelengths. A 26% quench in fluorescence at 350nm is observed on addition of α -santonin (0.2 μM) to the shock fluid as shown in Fig. 3 (A), thereby confirming the specific protein ligand binding

Fig. 1 Scanning densitogram of SDS PAGE of whole cell protein profile of α -santonin grown cells (A), freeze thawed cells (B) and shock fluid (C).

correlating with the linear regression curve shown in Fig. 3(B).

Uptake of α -santonin

The pattern of uptake of α -santonin in *S. paucimobilis* strain S cells as seen in previous report¹³ and in (Fig. 4a) appears to be biphasic. The initial phase, herein referred to as Type I, occurring in 2–3 min of exposure of cells to substrate α -santonin with the rate of uptake being 25 $\mu\text{g}/\text{ml}$ is instantaneous and is affected by the EDTA, sodium azide and α , α 1-bipyridyl¹³. Surprisingly, the freezing and thawing does not affect Type I uptake of the substrate (Fig. 4a). In contrast, the latter slow uptake, Type II, occurring after

Fig. 2 SDS PAGE profile of whole cell protein profile of α -santonin grown cells (lane 1), freeze thawed cells (lane 2) and shock fluid (lane 3).

3 min at a rate of 9.1 and 5 $\mu\text{g}/\text{ml}/\text{min}$ at 15 min and 30 min is seen to be totally impaired in freeze thawed cells, (Fig. 4a). These observations indicate that two independent sequential events are involved in uptake of α -santonin as type I and type II phases. Such a biphasic uptake Kinetics has been reported previously²².

Restoration of such shock sensitive, Type II uptake activity on reconstitution of the freeze thawed cells with the shock fluid to 95% (Fig. 4a) implies the involvement of periplasmically located substrate binding proteins or transport factor, as has been reported earlier in the transport of phosphate²³, ribose²⁴, glutamine²⁵, β -galactoside²⁶, maltose²⁷ and sugars and amino acids^{14,28}.

Transformation of α -santonin

S. paucimobilis strain S cells incubated with α -santonin form an array of products (Fig. 4b), detectable by TLC^{7,9,10}.

Binding proteins are often associated with dehydrogenases in the periplasm²⁹. Reduction of α -santonin, 1,2 dihydrosantonin by saturation of 1, 2 double bond is catalysed

by NADPH dependant 1-2 α -santonin dehydrogenase⁹. The shock fluid however failed to show α -santonin dehydrogenase activity as monitored by the decrease in absorbance of NADPH at 340 nm and it also failed to show any transfor-

Fig. 3 (A) Emission scan of shock fluid, shock fluid with α -santonin. The quench in fluorescence at 350nm indicated by the arrow.

(B) Linear regression curve of substrate concentration vs fluorescence intensity of periplasmic proteins.

mation products. This clearly indicates the involvement of the released proteins only in transport. The freeze thawed cells formed a single feeble product, less polar than α -santonin, at RF 0.57, (Fig. 4b) corresponding to lumisantonin⁹. The same cells however, when suspended in shock fluid, showed complete transformation of α -santonin, as with normal cells (Fig. 4b).

Identification of Santonin binding Protein

Periplasmic substrate binding protein is the unique feature of bacterial ABC importers³⁰. These binding proteins, of which there are dozens of different species in *E.coli* are released into the medium by osmotic shock; their absence results in the loss of ability to transport their specific substrates. Binding proteins undergo a chemical change in its chemical environment upon binding with substrate which causes a quench or enhancement in fluorescence intensity³⁰. As we have seen earlier our results from transport assay, transformation, reconstitution experiments and the fluorescence spectroscopy results have demonstrated the same indicating the involvement of the protein in transport and undergoing a conformational change. Studies related to characterisation

Fig. 4 (A) Uptake of α -santonin by *S. paucimobilis* strain S Cells (●); freeze thawed cells (X), thawed cells reconstituted in shock fluid (⊙). Uptake of α -santonin was measured by estimating the amount of undepleted α -santonin in the filtrate of cells.

(B) Transformation of α -santonin (D) by *S. paucimobilis* strain S Cells (A); freeze thawed cells (B), thawed cells reconstituted in shock fluid (C). (B) Thin layer chromatogram of chloroform extracts of cells incubated with α -santonin. $\odot \rightarrow \bullet$ spots indicate increasing intensity.

of periplasmic binding proteins were conducted by studying electrophoretic mobility of these proteins on a nondenaturing gel electrophoresis. The change in mobility of the corresponding protein was observed and on elution from the gel its molecular weight was determined and was confirmed to be a 46 kDa from the shock fluid. Several such investigations need to be undertaken as it would help us to propose the role played by ABC transporter (traffic ATPases) in transport of α -santonin across the cell envelope of *S. paucimobilis*.

Repeated freezing and thawing of *Sphingomonas* strain S ATCC 43388 released shock fluid with an ability to bind α -santonin contributing to a 26% quench in fluorescence at 350 nm. The shock fluid also restores the impaired uptake and transformation of thawed cells to near original level. Hence it is pertinent to deduce that the α -santonin uptake/transport is governed by the binding proteins/ permeases located in the periplasmic space of the cell.

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