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S-(*p*-Azidophenacyl)thiocarnitine specifically binds to the γ-butyrobetaine-binding protein of *Agrobacterium* sp. and can be used as a photoaffinity label

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The photoaffinity reagent *S*-(*p*-azidophenacyl)thiocarnitine (PAP-TC) has been synthesized according to Mauro et al. [(1986) Biochem. J. 237, 533–540]. This compound, originally designed for a structure-function study of carnitine acetyltransferase, was used to analyze the *Agrobacterium* sp. γ-butyrobetaine transport system. PAP-TC appears to be a reagent specific to the transport system since it showed a competitive inhibition ($K_i = 70$ μM) of γ-butyrobetaine transport. UV irradiation of periplasmic proteins in the presence of [^{14}C]PAP-TC resulted in the irreversible labeling of the γ-butyrobetaine-binding protein. The addition of 1 mM γ-butyrobetaine in the mixture significantly decreased the incorporation of the reagent, showing that this compound reacts specifically with the binding protein.

Photoaffinity labeling; Protein labeling; Binding protein; Transport; (*Agrobacterium*)

1. INTRODUCTION

Agrobacterium sp. has been isolated from soil and can use γ-butyrobetaine (fig.1a) as its sole source of carbon, nitrogen, and energy. This bacterium possesses an inducible transport system for this substrate, which involves a periplasmic γ-butyrobetaine-binding protein [1]. In order to analyze the substrate specificity of this protein, it would be interesting to specifically label the recognition site, isolate responsible peptides, and compare their structure with other proteins involved in the binding or metabolism of carnitine derived compounds. This paper reports the utilisation of an analogue of γ-butyrobetaine as a photoaffinity label for the γ-butyrobetaine-binding

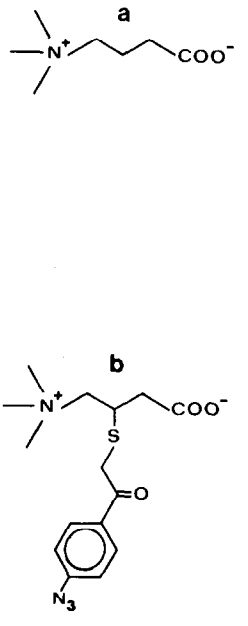

Fig.1. Structure of γ-butyrobetaine (a) and of the photolabel probe PAP-TC (b).

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PAP-TC, *S*-(*p*-azidophenacyl)thiocarnitine

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protein. We demonstrate that this compound (PAP- TC, fig.1b), originally designed for the photoaffinity labeling of carnitine acetyltransferase [2], inhibits γ-butyrobetaine transport in *Agrobacterium* sp. by interaction with the periplasmic binding protein. After photoirradiation in the presence of [^{14}C]PAP-TC, we were able to label specifically the γ-butyrobetaine-binding protein.

2. MATERIALS AND METHODS

2.1. *Chemical synthesis*

γ-[^{14}C]Butyrobetaine was synthesized as described [1]. PAP-TC was synthesized by coupling thiocarnitine [3] with the photolabile ligand *p*-azidophenacylbromide [2]. Purification was performed by preparative TLC (cellulose-coated plate without fluorescent indicator) and detection by exposing the plate to I_2 vapour. Synthesis of [^{14}C]PAP-TC was carried out with DL-[^{14}C]carnitine hydrochloride (10 μCi/μmol) as starting material and essentially as described [2,3], with the exception that H_2SO_4 was omitted in the first step of the reaction. This modification greatly increased the yield of the synthesis. [^{14}C]PAP-TC was detected on TLC plates either radioautographically or with a radiochromatogram scanner (Berthold, mod. 2832).

2.2. *Periplasmic protein preparation*

Agrobacterium sp. strain HK_4 (DSM2938) was grown at 30°C with γ-butyrobetaine as carbon and nitrogen sources [1] and periplasmic proteins were obtained by osmotic shock [4]. These proteins were concentrated by ultrafiltration and extensively dialyzed against 10 mM Tris-HCl, pH 7.3. A detail purification of the γ-butyrobetaine-binding protein will be presented in a future communication.

2.3. *Binding assay*

To determine the K_d, the non-equilibrium dialysis technique was used [5]. Two small dialysis tubes were closed at one end and fitted at the other with a shortened plastic pipette tip. 190 μl of 10 mM Tris-HCl, pH 7.3, either with or without pure γ-butyrobetaine-binding protein (3 mg/ml) were added, followed by 10 μl of [^{14}C]PAP-TC (approx. 6 μM). Aliquots of 20 μl were removed at various times from both bags during dialysis against 10 mM Tris-HCl, pH 7.3, and counted for radioactivity. K_d was calculated as described [6].

2.4. *Determination of the inhibition constant (K_i) of γ-butyrobetaine transport by PAP-TC*

K_i was calculated from the Lineweaver-Burk plot of γ-[^{14}C]butyrobetaine transport activity in the presence or absence of 100 μM PAP-TC [7].

2.5. *Photoaffinity labeling experiments*

Labeling of the purified γ-butyrobetaine-binding protein or the crude periplasmic proteins was carried out at a protein and [^{14}C]PAP-TC concentration of 1 mg/ml and 50 μM, respectively. Irradiation, for 10 min was as described [2]. Samples were analyzed by 12.5% acrylamide SDS-PAGE [8]. The gel was stained with Coomassie blue and autoradiographed. Protein content was determined by the method of Bradford [9].

3. RESULTS AND DISCUSSION

3.1. *Inhibition of γ-butyrobetaine transport by PAP-TC*

Cells were first fully induced for γ-butyrobetaine transport and then the transport of γ-[^{14}C]butyrobetaine at concentrations ranging from 0.3 to 6 μM in the presence of either 100 or 700 μM PAP-TC was measured (fig.2 shows the Lineweaver-Burk plot obtained with 100 μM PAP-TC). Both concentrations yielded the same pattern of inhibition and the same value for the K_i, i.e., 70 μM, demonstrating that the inhibition by this compound is fully competitive.

3.2. *Binding of [^{14}C]PAP-TC to the γ-butyrobetaine-binding protein*

The competitive inhibition observed using intact cells suggested that the inhibitory effect of PAP-TC could be directly on the binding protein. Therefore, we measured the binding affinity of PAP-TC for the γ-butyrobetaine-binding protein. We determined the rate at which [^{14}C]PAP-TC left the dialysis bag in the presence or absence of γ-butyrobetaine-binding protein. In non-equilibrium

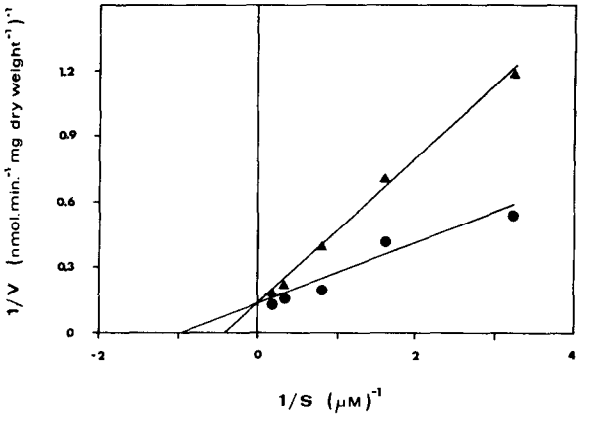

Fig.2. Kinetics of γ-butyrobetaine uptake by *Agrobacterium* sp. strain HK_4 in the presence (▲) or absence (●) of 100 μM PAP-TC (no photolysis). Uptake was measured by filtering 0.5 ml cell samples through Sartorius filters after a 15 s incubation in the presence of 0.3 -6 μM γ-[^{14}C]butyrobetaine.

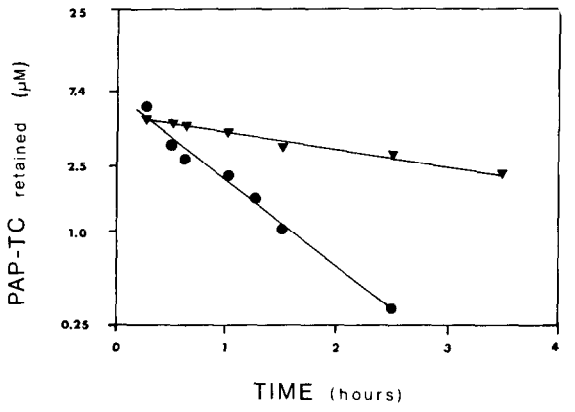

Fig.3. Retention of [^{14}C]PAP-TC by the γ-butyrobetaine-binding protein. Dialysis bags were filled with 200 μl of 10 mM Tris-HCl, pH 7.3 containing about 6 μM radioactive PAP-TC in the presence or absence of γ-butyrobetaine-binding protein. The bags were then dialysed against 2 l of buffer and aliquots of 20 μl were removed and counted. Experiment with (▼) and (●) without γ-butyrobetaine-binding protein.

dialysis and at a given protein concentration, the K_d can be calculated by following the rate of the loss of [^{14}C]PAP-TC from the dialysis bag. These results are shown in fig.3. In the presence of the binding protein, the exit rate is 4.7 times slower than in its absence. This difference corresponds to a K_d of 15 μM, 15 times higher than the K_d for γ-butyrobetaine [1]. Therefore, it seems that the inhibition of γ-butyrobetaine uptake is the consequence of an interaction with the binding protein. However, an interaction with the external membrane cannot be ruled out because of the different values we obtained for the K_i and K_d. When [^{14}C]PAP-TC was used as substrate in a transport assay with whole cells, no accumulation was detected (not shown). Thus it seems that PAP-TC is not recognized by membrane-bound components of the transport system and cannot be translocated into the cell.

3.3. *Photoaffinity labeling of the γ-butyrobetaine-binding protein*

In order to demonstrate that PAP-TC can bind irreversibly to the γ-butyrobetaine-binding protein upon photolysis [^{14}C]PAP-TC was photoactivated in the presence of either crude osmotic shock fluid or purified binding protein. The mixture was then subjected to SDS-PAGE followed by autoradiography. The Coomassie-stained gel and the corresponding autoradiograph are shown in fig.4. γ-Butyrobetaine-binding protein was labeled only when γ-butyrobetaine was absent from the media. Labeling of the γ-butyrobetaine-binding protein also occurred in the crude osmotic shock fluid extract. This experiment demonstrates that the azide-based photoprobe has formed a covalent bond. Having demonstrated that the γ-butyrobetaine-binding protein can be specifically labeled using PAP-TC, we intend to use this compound as a probe for a structure-function study of this protein.

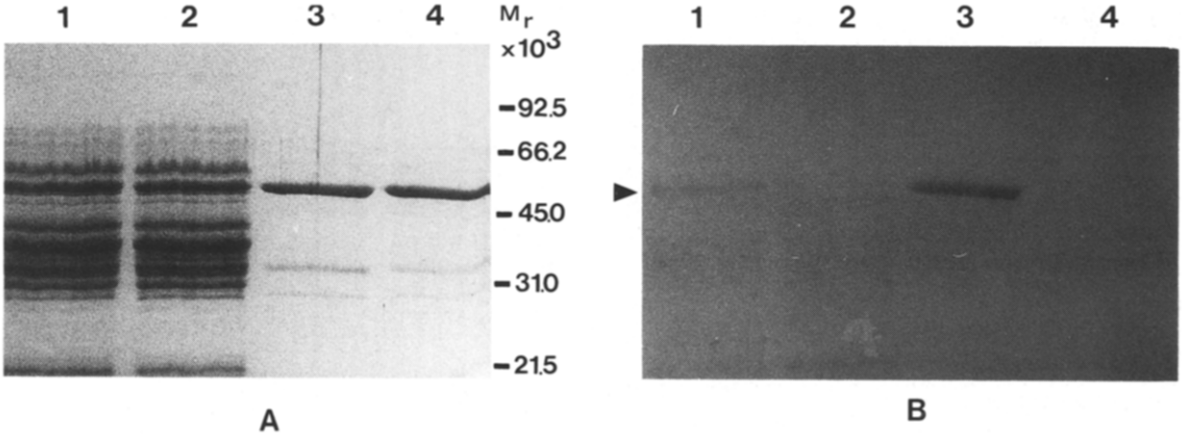

Fig.4. Labeling of γ-butyrobetaine-binding protein by [^{14}C]PAP-TC. Crude osmotic shock fluid (lanes 1 and 2) or purified γ-butyrobetaine-binding protein (lanes 3 and 4) were incubated with [^{14}C]PAP-TC and irradiated at 366 nm prior to electrophoresis on SDS-PAGE. The preparations shown in lanes 2 and 4 were done in the presence of 1 mM γ-butyrobetaine. (A) Gel; (B) autoradiogram of the gel shown in (A). Arrow shows the position of the γ-butyrobetaine-binding protein.

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