Asp-285 of the metal-tetracycline/H^+ antiporter of *Escherichia coli* is essential for substrate binding

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Abstract The transposon Tn10-encoded metal-tetracycline/H^+ antiporter (TetA(B)) was preferentially photolabeled when [^{3}H]tetracycline was irradiated in the presence of energized inverted membrane vesicles containing the TetA protein. The degree of labeling depended on the duration of irradiation and the energization of the membrane. Photolabeling was not observed in vesicles containing the Asp-285 →Asn mutant TetA protein, indicating that Asp-285 participates in the substrate binding or the step(s) prior to substrate binding

Key words: Tetracycline; Tetracycline/H^+ antiporter; Tet; Photolabel; Subtrate binding

1. Introduction

The metal-tetracycline/H^+ antiporter (TetA) is a member of a major facilitator family of integral membrane proteins that have 12 membrane-spanning segments [1], which includes not only drug exporters [2,3] but also many kinds of secondary transporters of sugars, amino acids, neurotransmitters and organic monoamines. The TetA proteins are important as a paradigm for studying the molecular mechanism of this major facilitator family since they are the only well-studied antiporters in this family. We have performed several site-directed mutagenesis studies on the molecular mechanism of the typical TetA protein encoded by transposon Tn10 (TetA(B)) [4–8]. The TetA(B) protein contains three aspartate residues in the putative transmembrane domain, which are conserved throughout the TetA proteins of Gram-negative bacteria [5]. These aspartate residues had been estimated to participate in substrate recognition or translocation across the membrane since the substrate of the TetA protein is a monocationic tetracycline-divalent cation chelation complex [9]. Site-directed mutagenesis of these aspartate residues confirmed the importance of these residues [5]. Especially, Asp-285, which is located on the middle of the transmembrane helix 9, is essential for the function.

The Asp-285→Asn mutant of the TetA protein has completely lost tetracycline resistance and transport activity [5]. However, the mutation was suppressed by a second-site mutation acquiring a carboxyl group at position 220 [10], indicating the importance of a negatively charged carboxyl group in the process of tetracycline transport. In order to determine the role of this essential carboxyl group in the transport process, it is necessary to detect the effect of the mutation on each elementary step of the transport. Unfortunately, the tetracycline-bound form of the TetA protein has not been detected because the binding of a substrate to the TetA protein is only transient and very unstable.

Photo-activatable analogues are available to fix the substrate binding state of transport proteins such as P-glycoproteins [11,12]. The tetracycline molecule is photoreactive and the technique of photolabeling has been used for determining the tetracycline binding site on *E. coli* ribosomes [13,14]. In this study, we attempted photolabeling of the TetA protein by radioactive tetracycline in order to detect the effect of the Asp-285→Asn mutation on substrate binding.

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2. Materials and methods

2.1. Materials

[7-^{3}H(N)]Tetracycline (17.0 GBq/mmol) was purchased from DuPont-New England Nuclear. All other materials were of reagent grade and obtained from commercial sources.

2.2. Construction of mutant plasmids

Plasmid pSYTET carries the *tet*A gene downstream of the *lac* promoter, resulting in overproduction of TetA protein [15]. The *Eco*RI-*Bam*HI DNA fragment of pTBD285N [5], which carries the Asp-285→Asn mutation, was exchanged with the corresponding fragment of pSYTET. The resultant plasmid was named pSYD285N.

2.3. Preparation of everted vesicles and separation of inner membrane vesicles

Everted membrane vesicles were prepared from *E. coli* W3104 [16] harboring a low-copy-number plasmid, pLGT2 [5], and *E. coli* RB791 harboring a high-copy-number plasmid, pSYTET or pSYD285N, after induction of *tet*A gene expression by 2 h incubation with 0.25 μg/ml heat-inactivated chlortetracycline [17] and 0.1 mM IPTG, respectively, using a French press as described previously [18]. The inner membrane fraction was separated from the outer membrane fraction by sucrose density gradient centrifugation [19]. The inner membrane vesicles were collected by ultracentrifugation and resuspended in 50 mM Tris-HCl buffer (pH 7.6) containing 50 mM KCl. The resultant vesicle suspension was stored at −80°C.

3. Results and discussion

3.1. Photolabeling of the TetA protein by [^{3}H]tetracycline

Everted membrane vesicles were prepared from *E. coli* W3104 cells harboring a low-copy-number plasmid, pLGT2, after 2 h induction of the *tet*A gene expression as described under Section 2. The vesicles (250 μg membrane protein) were incubated in 50 μl of 50 mM Tris-HCl buffer (pH 7.6) containing 50 mM KCl, 10 mM $MgCl_2$ and 12.5 mM β-NADH for 1 min at room temperature, then [^{3}H]tetracycline (final concentration 100 μM) was added to the mixture. After irradiation with 365 nm UV light for the indicated periods with shaking, the mixture was diluted with 900 μl of the same buffer containing 1 mM unlabeled tetracycline in place of [^{3}H]tetracycline and β-NADH in order to stop the labeling.

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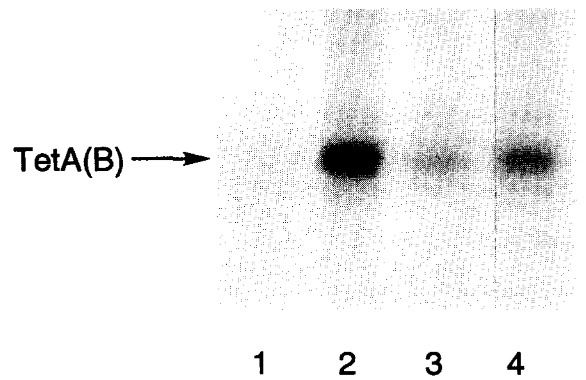

Fig. 1. Photolabeling of the TetA protein with [^{3}H]tetracycline. TetA proteins were solubilized from everted membrane vesicles of *E. coli* W3104/pLGT2 and collected by immunoprecipitation after irradiation of the vesicles for the indicated periods in the presence of [^{3}H]tetracycline and β-NADH as described in the text. Then SDS-polyacrylamide gel electrophoresis of the TetA proteins was performed followed by visualization of the radioactive bands with autoradiography after exposure for 140 days on X-ray film. Lanes: 1, vesicles incubated for 30 min in the presence of 100 μM [^{3}H]tetracycline and 12.5 mM β-NADH without irradiation; 2, vesicles irradiated under 365 nm UV light for 30 min in the presence of [^{3}H]tetracycline and β-NADH; 3, vesicles irradiated for 10 min under the same conditions as lane 2; 4, vesicles irradiated under the same conditions as lane 2 except for the absence of β-NADH.

Vesicles were collected and washed once with the same buffer by ultracentrifugation at 200 000 × g for 30 min at 4°C with a Beckman Optima TL ultracentrifuge. The resultant precipitate was solubilized in 200 μl of 10 mM sodium phosphate (pH 7.4) containing 0.1 M NaCl, 1% Triton X-100 and 0.1% SDS. Insoluble materials were removed by ultracentrifugation at 200 000 × g for 30 min at 4°C. Then the resultant supernatant was mixed with 20 μl of anti-TetA-carboxyl-terminal peptide antiserum [20] followed by incubation at room temperature for 1 h with shaking. Then 100 μl of a Pansorbin *S. aureus* cell suspension (Carbiochem) [21] was added to the mixture and incubation was continued for a further 1 h. The immunoprecipitate was collected by centrifugation at 1000 × g for 10 min at room temperature, and then washed twice with 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl, 1% Triton X-100 and 0.1% SDS. SDS polyacrylamide gel electrophoresis of the precipitate was performed. The resultant gel was soaked in Amplify (Amersham) in order to enhance the radioactivity prior to drying. The dried gel was exposed on X-ray film for 140 days at −80°C.

As shown in Fig. 1, when the vesicles were irradiated with UV light for 30 min in the presence of [^{3}H]tetracycline and β-NADH, a dense radioactive band corresponding to the TetA protein (lane 2) was detected on autoradiography. The vesicles irradiated for 10 min (lane 3) showed a fainter band than those irradiated for 30 min. On the other hand, when the vesicles were not irradiated, there was no radioactive band (lane 1). These observations confirmed that [^{3}H]tetracycline was certainly incorporated into the TetA protein depending on the duration of UV light irradiation.

When the vesicles were irradiated for 30 min in the absence of β-NADH, the density of the radioactive band (lane 4) was significantly fainter compared to the vesicles irradiated in the presence of β-NADH, indicating that the photochemical binding of [^{3}H]tetracycline to the TetA protein depends on the energized state of the membrane. In our previous paper [22], we revealed that the TetA protein undergoes a conformational change depending on the presence of tetracycline and β-NADH as an energy source. These observations suggest that tetracycline might be occluded in the TetA protein in an energy-dependent manner.

3.2. *Effect of mutation at Asp-285 on the photochemical labeling of the TetA protein with [^{3}H]tetracycline*

In order to detect the photolabeled TetA protein directly on SDS electrophoresis gels of cell membranes without immunoprecipitation, we constructed the high-copy-number plasmid, pSYTET [15], in which the *tet*A gene was subcloned under the control of the *lac* promoter. In a previous paper [23], we reported that the TetA protein was overproduced in *E. coli* RB791/pSYTET when *tet*A gene expression was induced by IPTG. In this study, *tet*A gene expression from pSYTET was induced by 0.1 mM IPTG for 2 h. As shown in Fig. 2, a 36 kDa dense band corresponding to the TetA protein was observed on Coomassie Brilliant Blue staining (Fig. 2A, lane 2).

Inner membrane vesicles were prepared from *E. coli* RB791/pSYTET cells after 2 h induction with IPTG as described in section 2. The vesicles were then photolabeled with [^{3}H]tetracycline as described above in the presence of 12.5 mM β-NADH with UV light irradiation for 20 min. After washing the vesicles with the same buffer containing unlabeled tetracycline, the vesicles were directly solubilized in Laemmli sample buffer [24] followed by SDS gel electrophoresis. After 42 days exposure on X-ray film, the radioactive bands were visualized. As shown in Fig. 2B, a dense radioactive band was detected at 36 kDa (Fig. 2B, lane 1), which corresponds to the TetA protein. A minor radioactive band was also observed at about 29 kDa, which may be an unknown membrane protein having tetracycline binding ability. No other protein bands showed significant radioactivity. Therefore, it was confirmed that the photochemical labeling of [^{3}H]tetracycline of membrane proteins depends on the specific binding of tetracycline

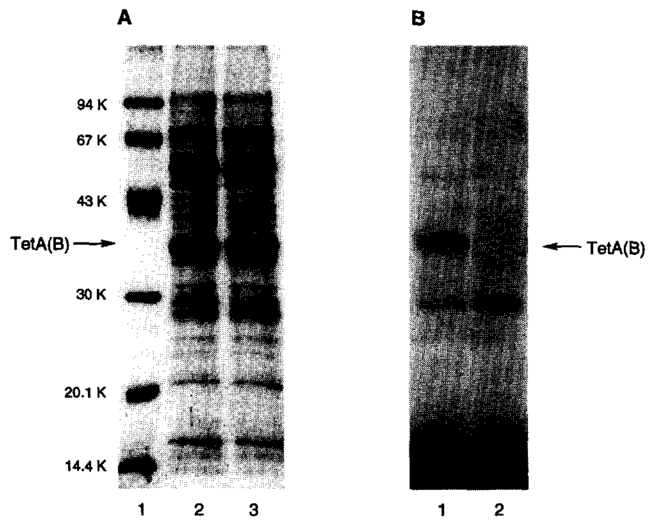

Fig. 2. SDS gel electrophoretic pattern of the inner membrane vesicles prepared from *E. coli* RB791 harboring a wild-type plasmid, pSYTET, or the D285N mutant plasmid, pSYD285N (A) and the radioactive bands visualized by autoradiography (B). (A) Coomassie Brilliant Blue staining. Lanes: 1, molecular weight markers; 2, pSYTET; 3, pSYD285N. (B) Autoradiography. The gel was exposed for 42 days on X-ray film. Lanes: 1, pSYTET; 2, pSYD285N.

to the proteins. Photolabeling was not due to non-specific random binding of tetracycline photoproduct.

Subsequently, we constructed the high-copy-number plasmid, pSYD285N, which encodes the Asp-285→Asn mutant TetA protein, by fragment exchange between pSYTET and pTBD285N [5]. The mutant TetA protein was expressed in *E. coli* RB791/pSYD285N and the inner membrane vesicles were prepared as in the case of the wild-type vesicles. The SDS-polyacrylamide gel electrophoresis pattern of the mutant vesicles (Fig. 2A, lane 3) was the same as in the wild-type vesicles, indicating that the mutation at position 285 did not affect gene expression. In contrast, the autoradiographic pattern of the mutant vesicles (Fig. 2B, lane 2) showed no radioactive band at 36 kDa corresponding to the TetA protein. The 29 kDa radioactive band of the unknown protein was observed in mutant vesicles similar to that of the wild-type vesicles, therefore, the photochemical reaction certainly took place in mutant vesicles. These results clearly indicate that the photochemical labeling of the TetA protein occurs only after binding of tetracycline to the substrate recognition site and the Asp-285→Asn mutant TetA protein lost the tetracycline binding ability.

The carboxyl group of Asp-285 is essential for the transport function of the TetA protein [5]. The Asp-285→Asn mutant has no tetracycline transport activity [5] and the activity was recovered on the acquisition of a carboxyl group at position 220 by a second-site suppressor mutation [25]. Judging from the present results, Asp-285 participates in the substrate binding step or the step(s) before the substrate binding in the substrate translocation process through the TetA protein.

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