Substrate-induced acceleration of *N*-ethylmaleimide reaction with the Cys-65 mutant of the transposon Tn10-encoded metal-tetracycline/H^+ antiporter depends on the interaction of Asp-66 with the substrate

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Received 7 February 1995; revised version received 20 February 1995

Abstract We previously reported that the reaction of [^{14}C]*N*-ethylmaleimide (NEM) with the S65C mutant of the transposon Tn10-encoded metal-tetracycline/H^+ antiporter (TetA(B)) is competitively inhibited by tetracycline [Yamaguchi, A. et al., FEBS Lett. 322 (1993) 201–204]. However, this observation has been revealed to be a mistake. The reaction of [^{14}C]NEM with S65C TetA(B) was significantly and reproducibly accelerated by tetracycline, i.e. not inhibited. When Asp-66 was replaced by Ala, the reaction of NEM with the Cys-65 residue was no longer affected by tetracycline. In contrast, when Arg-70 was replaced by Ala, the acceleration of the reaction was unaltered. The tetracycline acceleration of the reaction to the Cys-65 residue was further stimulated with energization of the membrane on the addition of NADH. On the other hand, the tetracycline-induced acceleration was not observed in the absence of a divalent cation. These observations indicated that the Cys-65 locus is exposed to the medium according to the interaction of a divalent cation-tetracycline chelation complex with Asp-66.

Key words: Antiporter; Tetracycline/H^+ antiporter; Site-directed mutagenesis; Sulfhydryl reagent

1. Introduction

The transposon Tn10-encoded metal-tetracycline/H^+ antiporter (TetA(B)) contains a highly conserved sequence motif, GXXXDRXGRR, in the putative cytoplasmic loop$_{2-3}$ [2]. This motif is conserved not only in the bacterial drug/H^+ antiporters but also in the bacterial solute/H^+ symporters [3] and mammalian glucose transporters [4]. However, the precise role of this motif remains unknown. We first studied the role of this motif by means of site-directed mutagenesis [2,5]. As a result, it was revealed that two glycines in this motif are important for maintenance of the loop structure [2]. In particular, Gly-69 is related to the b-turn structure of this loop. Asp-66 is the only acidic residue in this loop and the negative charge of its carboxyl side chain is essential for the function. The positive charge of Arg-70 is also important for the function but the other two conserved arginines, Arg-67 and Arg-71, are not important for the activity. Ser-65 is the residue just before the essential Asp-66 but is not important for the function [5]. However, when Ser-65 was replaced by Cys, the resulting S65C mutant was completely inactivated by *N*-ethylmaleimide (NEM) [5], whereas the activity of neither the wild-type TetA(B) nor the other Cys mutants of this loop was affected by NEM [2]. The degree of the inactivation of the S65C mutant by sulfhydryl reagents depended on the volume of the substituent [6], suggesting that the inactivation is due to steric hindrance by the substituent. This was confirmed by the observation that the activity of the site-directed TetA(B) mutants was inversely proportional to the side chain volume of the residue at position 65 [6]. Finally, we observed that the reaction of [^{14}C]NEM with the S65C mutant was competitively inhibited in the presence of tetracycline in the previous studies [1,6]. However, the last observation was a mistake. Later, we found that the reaction of [^{14}C]NEM with the S65C mutant was not inhibited by tetracycline but rather significantly accelerated. In this study, we investigated the cause of the acceleration of the reaction of NEM by the substrate.

2. Materials and methods

2.1. Materials

N-Ethyl[2,3-^{14}C]maleimide (333 MBq/mmol) and [7-^{3}H(N)]tetracycline (23.7 GBq/mmol) were purchased from Amersham and DuPont-New England Nuclear, respectively. All other materials were of reagent grade and obtained from commercial sources.

2.2. Plasmids

pCT1183 [7] and pLGT2 [8] are high copy number and low copy number plasmids, respectively, containing *tet*A(B) and *tet*R(B) genes cloned from transposon Tn10 [9]. pERS65C was constructed by subcloning a 485 bp *Eco*RV–*Eco*RI fragment of pLGS65C [5], which carries the S65C mutant *tet*A(B) gene, into pUC118RV [8], in which the *Sac*I site of pUC118 [10] had been changed to an *Eco*RV site. In the resultant plasmid, the 5′-terminal half of the *tet*A(B) gene was cloned in the reverse direction.

2.3. Construction of S65C/D66A and S65C/R70A double mutants, and a D66C single mutant by site-directed mutagenesis

Mutagenesis was performed by the oligonucleotide-directed site-specific mutagenesis method of Kunkel [11]. Oligonucleotides were synthesized with a Cyclone Plus DNA/RNA Synthesizer (MilliGen Biosearch Co.). The S65C/D66A double mutant was constructed using pERS65C, as a template, and a synthetic primer, 5′-AAATGTGCGCGCGATTTGGT-3′, which corresponds to the coding sequence containing mismatches causing Ser-65 → Cys and Asp-66 → Ala mutations. The mutation was at first detected as the disappearance of the *Pvu*I site, which exists in the corresponding sequence of pERS65C. After determination of the DNA sequence, the *Eco*RV-*Eco*RI fragment of the resultant plasmid was transferred to pLGT2 by corresponding fragment exchange in order to construct pLGS65C/D66A. The S65C/R70A double mutant was constructed in a similar manner to the S65C/D66A mutant using pERS65C, as a template, and a synthetic primer, 5′-GATTTGGAGCTCGCCCAGTGC-3′, which contains mismatches causing a Arg-70 → Ala mutation and silent mismatches generating a *Sac*I site, and reconstructed to pLGS65C/R70A by fragment exchange. The mutation was detected as the appearance of the *Sac*I site. Finally, the D66C single mutant was constructed using pCT1183, as a template, and a synthetic oligomer having the complementary sequence of 5′-ACC-

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SSDI 0014-5793(95)00205-7

AAATCTGCAGGACATTTT-3′, which contains mismatches causing a Asp-66→Cys mutation and generating a *PstI* site. The mutation was detected as the appearance of the *PstI* site and, after DNA sequencing, pLGD66C was constructed by corresponding fragment exchange.

2.4. Preparation of inverted membrane vesicles and transport assaying

Inverted membrane vesicles were prepared from *Escherichia coli* W3104 [12] cells carrying pLGT2 or its derivatives encoding TetA(B) mutants, as described previously [5]. [^{3}H]Tetracycline uptake by inverted membrane vesicles was assayed in the presence of 10 μM [^{3}H]tetracycline and 50 μM $CoCl_2$ in 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl [13].

2.5. Assaying of the reaction of [^{14}C]N-ethylmaleimide with TetA(B) proteins

Inverted membrane vesicles (0.5 mg protein) were incubated in 100 μl of 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl, 5 mM $MgSO_4$ and 0.5 mM [^{14}C]NEM at 30°C for 1 min or 5 min in the presence or absence of 1 mM tetracycline. When the vesicles were energized, they were pretreated with 50 mM NADH for 1 min at 30°C prior to the addition of [^{14}C]NEM and tetracycline. Immediately after the incubation with [^{14}C]NEM, the vesicles were diluted with 900 μl of 50 mM MOPS-KOH buffer (pH 7.0) containing 0.1 M KCl and excess unlabeled NEM (10 mM) to stop the reaction. Then the vesicles were precipitated by ultracentrifugation at 200,000 × *g* for 30 min at 4 °C in a Beckman ultracentrifuge, Optima TL. The resultant precipitate was solubilized in 200 μl of 10 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl, 1% Triton X-100, 0.1% SDS and 5 mM unlabeled NEM by brief sonication in a Branson bath sonifier. Insoluble substances were removed by ultracentrifugation at 200,000 × *g* for 30 min at 4°C. The resultant supernatant was mixed with 15 μl of anti-TetA-carboxyl-terminal antiserum [14] in an Eppendorf tube, followed by incubation at room temperature for 1 h with shaking. Then 120 μl of a Pansorbin *S. aureus* cell suspension (Carbiochem) [15] was added to the mixture and the incubation was continued for a further 1 h. The immunoprecipitate was collected by centrifugation at 1,000 × *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 gel electrophoresis of the precipitate was performed, and the radioactive band was visualized by fluorography and quantitated with a Bioimaging Analyzer BAS2000 (Fuji Film Co., Tokyo).

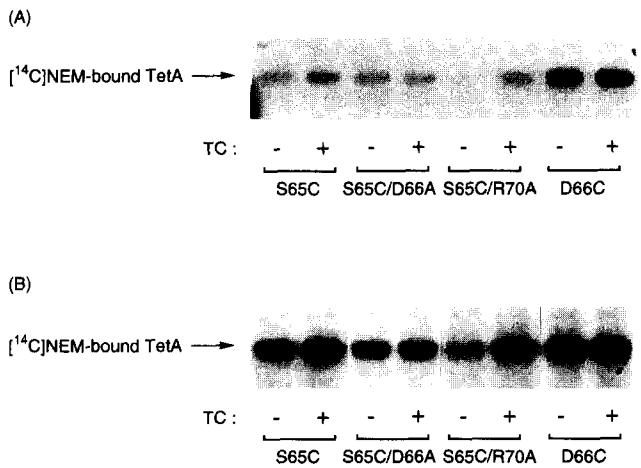

Fig. 1. The effect of Mg-tetracycline on the [^{14}C]NEM binding to TetA(B) mutants. Inverted membrane vesciles (0.5 mg protein) containing the S65C, S65C/D66A, S65C/R70A for D66C mutant TetA(B) protein were incubated with 0.5 mM [^{14}C]NEM in the absence or presence of 1 mM tetracycline and 5 mM $MgSO_4$ at 30°C for 1 min (A) or 5 min (B). The binding reaction was terminated by the addition of excess non-radioactive NEM, followed by solubilization and immunoprecipitation of the TetA(B) proteins as described in section 2. After SDS-PAGE, the yield gels were exposed to X-ray film for 40 days at −80°C. − and + indicate the absence and presence of Mg-tetracycline, respectively.

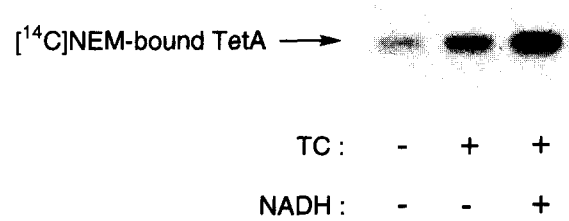

Fig. 2. The effect of membrane energization on the [^{14}C]NEM binding to the S65C mutant TetA(B). After 1 min preincubation with or without 50 mM NADH, inverted membrane vesicles (0.5 mg protein) containing the S65C mutant TetA(B) were incubated with 0.2 mM [^{14}C]NEM in the absence or presence of 2 mM tetracycline at 30°C for 5 min. Radioactive bands were visualized as described in Fig. 1.

3. Results and discussion

3.1. Effect of tetracycline on the reaction of [^{14}C]NEM with the S65C mutant TetA(B) protein

Inverted membrane vesicles containing the S65C mutant TetA(B) proteins were incubated with 0.5 mM [^{14}C]NEM for 1 min or 5 min in the absence or presence of 1 mM tetracycline and 5 mM $MgSO_4$. After stopping the reaction of [^{14}C]NEM by the addition of excess non-radioactive NEM, the vesicles were solubilized with detergents, followed by immunoprecipitation of TetA(B) proteins with an anti-C-terminal antibody [14]. [^{14}C]NEM-bound TetA(B) proteins were detected by autoradiography after SDS-PAGE and quantitated with a Bioimaging Analyzer BAS2000 (Fuji Film Co., Tokyo). As shown in Fig. 1A, the degree of the [^{14}C]NEM reaction with the S65C mutant after 1 min incubation in the presence of Mg-tetracycline was at least 2-fold higher than that in the absence of the substrate. The ratio of the degree of the reaction in the presence of tetracycline to that in its absence was roughly maintained after 5 min incubation (Fig. 1B), although the degree of the reaction in both cases increased during this period. Therefore, the effect of Mg-tetracycline was on the rate of the reaction of [^{14}C]NEM with the S65C mutant, suggesting that the substrate-induced conformational change of the TetA(B) protein caused the exposure of the SH group of Cys-65 to the medium.

In the previous paper [1], we reported that the reaction of [^{14}C]NEM with the S65C mutant was inhibited by tetracycline, however, the previous observation was a mistake due to the unexpected loss of the TetA protein during immunoprecipitation after tetracycline treatment. In the present study, each experiment was performed at least twice, and the immunoprecipitated TetA proteins were detected with Coomassie brilliant blue staining after SDS-PAGE of aliquots of each sample. In addition, the acceleration of the reaction was reproducibly observed in the different concentrations of tetracycline.

As a control, the effect of Mg-tetracycline on the reaction of [^{14}C]NEM with the D66C mutant was investigated. Although Cys-66 is the residue next to Ser-65, the reaction with the former was not affected by Mg-tetracycline (Fig. 1A and B). The D66C mutant has no tetracycline transport activity (data not shown), probably due to the lack of interaction with Mg-tetracycline. It is interesting that the rate of the reaction with the D66C mutant was much higher than that to the S65C mutant, indicating that the side chain at position 66 is sufficiently exposed to the medium.

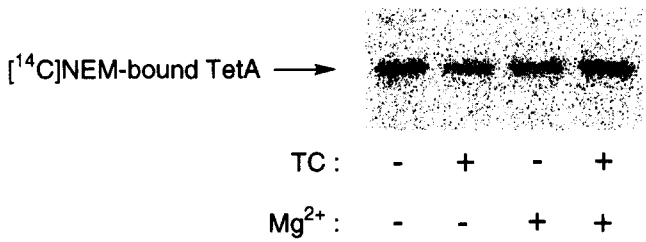

Fig. 3. The effect of magnesium ions on the [^{14}C]NEM binding to the S65C mutant TetA(B). Inverted membrane vesicles (0.5 mg protein) were incubated with 0.2 mM [^{14}C]NEM in the presence or absence of 1 mM tetracycline, with or without 5 mM $MgSO_4$, at 30°C for 5 min. Radioactive bands were visualized with a Bioimaging Analyzer BAS2000.

3.2. *Effect of mutations at Asp-66 and Arg-70 on the substrate-accelerated reaction of [^{14}C]NEM with Cys-65*

Asp-66 of the S65C mutant TetA(B) was replaced by a neutral residue, alanine, by site-directed mutagenesis. As shown in Fig. 1, the reaction of [^{14}C]NEM with the resulting double mutant, S65C/D66A, was no longer affected by the presence of Mg-tetracycline. Irrespective of the presence of tetracycline, the degree of the reaction with the S65C/D66A double mutant was intermediate between those to the S65C mutant with and without tetracycline. This finding indicated that the conformational change of the Cys-65 locus is probably due to the interaction of Mg-tetracycline with the negatively-charged carboxyl side chain of Asp-66. The replacement of Asp-66 with a neutral residue may fix the protein conformation between the conformations in the absence and presence of the substrate.

On the other hand, the substrate-induced acceleration of the reaction did not disappear on the replacement of Arg-70 by Ala, which is another important charged residue in $loop_{2-3}$ [2]. On the contrary, the degree of acceleration of the reaction appeared to be increased in the S65C/R70A double mutant, mainly due to the very low reactivity of the double mutant with NEM in the absence of tetracycline. There are two possibilities for such low reactivity, one is that the Cys-65 locus was more cryptic in the S65C/R70A double mutant than that in the S65C single mutant in the absence of the substrate or the other is that the basic side chain of Arg-70 facilitates deprotonation of Cys-65. In the latter case, Arg-70 may be sterically close to Cys-65 at least in the absence of the substrate. Anyway, it is clear that Arg-70 does not directly contribute to the substrate-induced conformational change of the Cys-65 locus.

The R70A and S65C/R70A mutants maintained very low tetracycline transport activity, i.e. about 3% of the wild type level, whereas the D66A and S65C/D66A mutants had no activity (data not shown). The loss of activity of the latter mutants is probably due to the lack of the substrate–protein interaction. In contrast, although the conformational change induced by the substrate–protein interaction was maintained in the former mutants, the transport activity was drastically decreased, probably due to the hindrance of the substrate translocation step after the initial substrate–protein interaction.

3.3. *The effect of membrane energization on the substrate-induced acceleration of the [^{14}C]NEM reaction*

TetA(B)-mediated tetracycline transport is driven by a proton motive force [16,17]. If the substrate-induced conformational change of the Cys-65 locus is a process of the substrate translocation reaction, it may be affected by a proton motive force. To investigate this possibility, inverted membrane vesicles were energized by NADH-dependent respiration prior to the addition of [^{14}C]NEM and tetracycline. As shown in Fig. 2, the substrate-accelerated reaction was really further stimulated by the addition of NADH. This finding suggests that the exposure of the Cys-65 locus is one of the processes of the substrate translocation reaction subsequent to the initial substrate–Asp-66 interaction.

3.4. *The divalent cation requirement for the acceleration of the [^{14}C]NEM reaction with the substrate*

The real substrate of TetA(B) is a Mg-tetracycline chelation complex [13]. As shown in Fig. 3, the acceleration of the reaction of [^{14}C]NEM with the S65C mutant with tetracycline was not observed if magnesium ions were not added. The magnesium ion itself did not affect the degree of the reaction of [^{14}C]NEM in the absence of tetracycline. This observation also confirms that the tetracycline-induced acceleration of the reaction of [^{14}C]NEM is due to the conformational change accompanying the initial substrate–protein interaction in the transport process.

The findings reported in this paper indicate that the conformational change in the loop2–3 locus induced on the interaction of Asp-66 with Mg-tetracycline is an initial step of the tetracycline translocation reaction.

Acknowledgements: This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education of Japan.

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