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Thiamin transport in *Escherichia coli*: the mechanism of inhibition by the sulfhydryl-specific modifier *N*-ethylmaleimide

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Abstract

Active transport of thiamin (vitamin B_1) into *Escherichia coli* occurs through a member of the superfamily of transporters known as ATP-binding cassette (ABC) transporters. Although it was demonstrated that the sulfhydryl-specific modifier *N*-ethylmaleimide (NEM) inhibited thiamin transport, the exact mechanism of this inhibition is unknown. Therefore, we have carried out a kinetic analysis of thiamin transport to determine the mechanism of inhibition by NEM. Thiamin transport in vivo exhibits Michaelis–Menten kinetics with $K_M = 15$ nM and $V_{max} = 46$ U mg^{-1}. Treatment of intact *E. coli* KG33 with saturating NEM exhibited apparent noncompetitive inhibition, decreasing V_{max} by approximately 50% without effecting K_M or the apparent first-order rate constant (k_{obsd}). Apparent noncompetitive inhibition is consistent with an irreversible covalent modification of a cysteine(s) that is critical for the transport process. A primary amino acid analysis of the subunits of the thiamin permease combined with our kinetic analysis suggests that inhibition of thiamin transport by NEM is different from other ABC transporters and occurs at the level of protein–protein interactions between the membrane-bound carrier protein and the ATPase subunit.

Keywords: Thiamin transport; ABC transporter; Sulfhydryl inhibition; Noncompetitive inhibition

1. Introduction

The bacterial ATP-binding cassette (ABC) family is one of the largest superfamilies of transporters (reviewed in Refs. [1–3]), whose most thoroughly characterized members include the maltose [4] and histidine [5] transporters. The genetic organization and structural characteristics of ABC transporters of Gram-negative bacteria are very similar in that they utilize a soluble substrate-binding protein that serves as the initial receptor, shuttling the substrate across the periplasmic space. The liganded periplasmic space binding protein interacts with an integral membrane-bound carrier protein stimulating the hydrolysis of ATP by the ATPase subunit [6,7]. The hydrolysis of ATP then drives the translocation of the substrate into the cytoplasm. A detailed kinetic analysis of traffic ATPases has been proposed [8,9].

The transport of thiamin into *Escherichia coli* occurs by active transport, as demonstrated by several investigators [10–12], and is mediated by a periplasmic space binding protein-dependent permease that is specific for thiamin [10]. The cloning of the *E. coli* thiamin periplasmic space binding protein [13] and the identification and cloning of the integral membrane-bound carrier protein and the ATPase subunit involved in thiamin uptake in *E. coli* (listed in Ref. [3]) and *Salmonella typhimurium* [14] have confirmed that the thiamin periplasmic permease is a member of the ABC superfamily of transporters. ABC transport is the primary mode of uptake for thiamin since osmotic shock, a process that releases the thiamin periplasmic space binding protein [13,15], results in an approximately 90% decrease in thiamin uptake. This loss of transport of thiamin can be corrected by the addition of exogenous thiamin binding protein (TBP) [11,12].

The ABC transport of thiamin is inhibited nearly 90% by the sulfhydryl-specific modifier *N*-ethylmaleimide (NEM) [16], consistent with the inhibition of other ABC transporters by NEM [17–20]. The inhibition of thiamin trans-

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port by a sulfhydryl-specific modifier suggests that a cysteine(s) may either be critical for thiamin transport or is present in a region of a protein that is integral to the transport process. Although it has been demonstrated for P-glycoprotein and maltose transport that NEM modification inhibits transport by preventing the binding of ATP to the ATPase domain [17,19], the exact mechanism by which NEM inhibits thiamin transport is not yet known. Therefore, to better understand the mechanism of thiamin transport and its inhibition by NEM, we have examined the kinetics of thiamin transport into *E. coli* in the presence and absence of NEM. Thiamin uptake, either in the presence or absence or NEM, occurs by active transport and exhibits Michaelis–Menten kinetics, which allows certain mechanistic constraints to be applied to this system [8,9]. The observed first-order rate constant for thiamin uptake and the K_M of transport are both independent of [NEM]. However, the V_{max} for transport decreased $\approx 50\%$ with increasing [NEM]. Therefore, combined with a primary amino acid analysis of the integral membrane-bound carrier protein and the ATPase subunit of the thiamin ABC transporter, our kinetic results are consistent with a model in which NEM modifies a thiol(s) in either the EAA-domain of the membrane-bound carrier protein or in the ABC signature domain of the ATPase subunit. Both of these domains are believed to be critical for mediating the interaction between the membrane-bound carrier protein and the ATPase subunit. Modification of either of these domains could conceivably inhibit thiamin transport by uncoupling ATP hydrolysis from the transport process.

2. Materials and methods

2.1. Materials

All water was prepared on a four-bowl Milli-Q water system including an Organex-Q cartridge (Millipore). NEM, chloramphenicol, thiamin hydrochloride, thiamin monophosphate chloride (TMP), and thiamin diphosphate chloride (TDP) were purchased from Sigma. [*Thiazole*-2-^{14}C]thiamin hydrochloride was purchased from Amersham. 4-Methylthiazole hydrochloride was prepared by neutralization of an aqueous solution of 4-methylthiazole (99%, Aldrich) with HCl, removal of the solvent by evaporation at 50 °C under a water aspirator vacuum, and recrystallization from absolute ethanol: mp 96–99 °C. All other reagents were the highest quality available from commercial sources and were used as received.

2.2. Culture methods

All transport experiments were performed with *E. coli* KG33 [21]. This strain is auxotrophic for 4-methylthiazole and overproduces proteins involved in thiamin transport [10]. Conditions for the growth and harvesting of this strain and its properties have been described in detail previously [10]. Thiamin transport was performed on this strain at 37 °C in M9 minimal medium (supplemented with 0.2% glucose).

2.3. Thiamin transport assay

Thiamin transport in the presence and absence of 0.1–1.0 mM NEM was assayed with intact *E. coli* KG33 by a modification of a previously described assay [10]. The cells were grown to saturation in 4 ml of M9 minimal medium (supplemented with 0.2% glucose and 0.6 μM 4-methylthiazole) and used to inoculate 100 ml of M9 minimal medium (supplemented with 0.2% glucose and 0.6 μM 4-methylthiazole) to 10^7 cells ml^{-1}. The cells were grown with aeration at 37 °C for 4 h (mid-log phase; $OD_{600} = 0.4$–0.6). The cells were harvested by centrifugation at 5500 × *g* for 10 min, washed twice with M9 minimal medium (supplemented with 0.2% glucose) and resuspended in 30 ml of the same medium (10^9 cells ml^{-1}). Ten milliliters of the suspension was transferred to each of three sterile polystyrene test tubes (17 × 100 mm), and warmed to 28 °C for 5 min in a rotary water bath shaker. 20–200 μl of 50 mM aqueous NEM was added to two of the 10-ml cell suspensions to obtain solutions containing 0.1–1.0 mM NEM. As a negative control, 100 μl of sterile H_2O was added to the third 10-ml cell suspension. The cell suspensions were incubated at 28 °C for 15 min, transferred to three 50-ml centrifuge tubes, centrifuged at 5500 × *g* for 10 min, and excess NEM was removed by washing the cell pellets twice with M9 minimal medium (supplemented with 0.2% glucose). Each of the three cell pellets was resuspended in 30 ml of M9 minimal medium (supplemented with 0.2% glucose) (10^8 cells ml^{-1}). Each 30-ml cell suspension was equally distributed (10 ml = 30 ml/3) between three sterile polystyrene test tubes (17 × 100 mm) for determination of transport in triplicate, and warmed to 37 °C for 10 min in a rotary water bath shaker. Four hundred microliters of 7.7 mM aqueous chloramphenicol was added to each cell suspension to final concentrations of 31 μM chloramphenicol and 10^8 cells ml^{-1}, and the cell suspension was incubated at 37 °C for 5 min.

Thiamin transport into chloramphenicol-treated *E. coli* KG33 was initiated by the addition of [*thiazole*-2-^{14}C]thiamin (24 Ci mol^{-1}). Transport was allowed to continue for a time t ($0 \le t \le 4$ h) before transport was stopped by filtering 500 μl of the cell suspension through a 0.45-μm HA nitrocellulose membrane (Millipore). The cell-laden nitrocellulose membranes were washed with 10 ml of M9 minimal medium (supplemented with 0.2% glucose), which was shown to remove thiamin that was not retained inside the outer membrane (data not shown). The cell-laden nitrocellulose membranes were dried, placed in a 22-ml glass scintillation vial containing 7 ml of EcoLite™ liquid scintillation cocktail, and counted in a Beckman LS-7500 scintillation counter for $\ge 10^4$ counts with automatic

quench control. Background counts (100–300 cpm) were determined with a nitrocellulose membrane treated as described above without cells. "Dry" cell weight (w_{cells}; 1–2 mg) in 500 μl of the cell suspension (treated as described above) was filtered through a tared 0.45-μm HA nitrocellulose membrane ($w_o \approx 26$ mg). The cell-laden nitrocellulose membrane was dried as described above, weighed on an analytical balance ($w_{obsd} \approx 27–28$ mg), and the dry cell weight calculated using $w_{cells} = w_{obsd} - w_o$. Total intracellular [*thiazole*-2-^{14}C]thiamin (pmol mg^{-1}) was determined as the total radioactivity (100–12,000 cpm) recovered on the nitrocellulose membrane (after subtraction of background counts) divided by the dry cell weight (mg). An activity unit for thiamin uptake is defined as the picomoles of thiamin accumulated per minute at 37 °C in M9 minimal medium (supplemented with 0.2% glucose).

2.4. Thiamin export assay

Thiamin export was assayed with intact, [*thiazole*-2-^{14}C]thiamin-loaded *E. coli* KG33 by a modification of a previously described assay [22]. Intact *E. coli* KG33 (10 ml of cell suspension; 10^9 cells ml^{-1}) were prepared as described above and incubated for 2 h at 37 °C with 3 μM [*thiazole*-2-^{14}C]thiamin (24 Ci mol^{-1}). The thiamin-loaded cells were harvested by centrifugation at 5500 × *g*, washed twice with M9 minimal medium, resuspended in 30 ml of M9 minimal medium (10^8 cell ml^{-1}), equally distributed (10 ml = 30 ml/3) between three sterile polystyrene test tubes (17 × 100 mm), and warmed to 28 °C for 10 min in a rotary water bath shaker. The cell suspensions were treated with 0.1–1.0 mM NEM and 31 μM chloramphenicol as described above except that all washes were performed with M9 minimal medium in the absence of supplemental glucose.

Thiamin export was initiated by the addition of 200 μl of 20% (w/v) glucose to give final concentration of 0.4% glucose that was allowed to react for a time *t* (0–2 h) before export was stopped by filtering 500 μl of each cell suspension through a 0.45-μm HA nitrocellulose membrane (Millipore). Total residual intracellular [*thiazole*-2-^{14}C]thiamin (pmol mg^{-1}) was determined and normalized for dry cell weight as described above.

2.5. Determination of free thiols in the periplasmic space thiamin binding protein (TBP)

All reagents were made fresh the day of the experiment, purged ≥ 30 s with nitrogen, and stored under a nitrogen atmosphere. SDS was added (0.2%) to solutions after purging with nitrogen and all experiments were performed under a nitrogen atmosphere. "Buffer" contained 50 mM aqueous sodium phosphate (pH 7.5) buffer containing 5 mM sodium chloride and 20 mM disodium EDTA. The periplasmic space TBP (270 μg; 8 nmol), purified as described previously [13], was mixed in a final volume of 1 ml in buffer in the presence and absence of 0.2% SDS at 25 °C in a cuvette with a 10-mm absorbance pathlength. The reaction was initiated by the addition of 35 μl of 10 mM dipotassium 5,5′-dithiobis(2-nitrobenzoate) (DTNB) and monitored by following the change in absorbance at 412 nm as a function of time in the thermostated cell compartment of a Hitachi U-2000 UV-visible spectrophotometer of which the photometric output was directed to a personal computer. The reaction was followed until it was complete (approximately 60 min). The number of thiol groups reacted was calculated using a molar extinction coefficient of 13,600 M^{-1} cm^{-1}. For the determination of the total thiol content of TBP, the reaction buffer contained 0.2% SDS in the presence and absence of 250 mM 2-hydroxy-1-ethanethiol (β-ME) and the protein was incubated at 37 °C for 30 min. β-ME was removed by passage of the reaction solution through a prepacked disposable Sephadex G-25 column (1.5 × 5 cm; Pharmacia) equilibrated with 25 ml of buffer containing 0.2% SDS. This procedure efficiently removed excess β-ME as confirmed by independent spectrophotometric analysis in the absence of any protein (data not shown). The reaction was initiated by the addition of DTNB as described above.

3. Results

Fig. 1 shows the time course for the accumulation of intracellular [*thiazole*-2-^{14}C]thiamin in *E. coli* KG33 at 37 °C after treatment with 0–0.5 mM mM NEM under conditions favoring thiol modification. The lines represent a computer generated best fit to a first-order accumulation of thiamin. Untreated *E. coli* KG33 accumulated [*thiazole*-2-^{14}C]thiamin to 3310 ± 110 pmol of thiamin per mg dry cell weight. Treatment of intact *E. coli* KG33 with 0.1 mM and 0.5 mM NEM decreased the extents of [*thiazole*-2-^{14}C]thiamin accumulation to 1750 ± 90 and 340 ± 20 pmol of thiamin per mg dry cell weight, respectively, consistent with previous reports [16]. Based on the intracellular volume and dry cell weight of *E. coli*, these results correspond to an approximate intracellular thiamin concentration of 50, 25, and 5 μM, respectively. This result therefore demonstrates that thiamin has been accumulated against a concentration gradient, either in the presence or absence of NEM, confirming the active transport of thiamin observed by others. However, despite a decrease in the total accumulation of thiamin, the observed first-order rate constant for the accumulation of thiamin, $k_{obsd}=(1.9 \pm 0.5) \times 10^{-4}$ s^{-1}, is independent of NEM treatment and [NEM] (Fig. 1 and Table 1). The half-life of 60 min ($=0.693/k_{obsd}$) for the accumulation of thiamin is in reasonable agreement with half-lives in the range 20–80 min reported for other periplasmic permeases [14,23,24].

The dependence of the initial rate for thiamin accumulation into NEM-treated and untreated intact *E. coli* KG33 (on thiamin) in M9 minimal medium (supplemented with

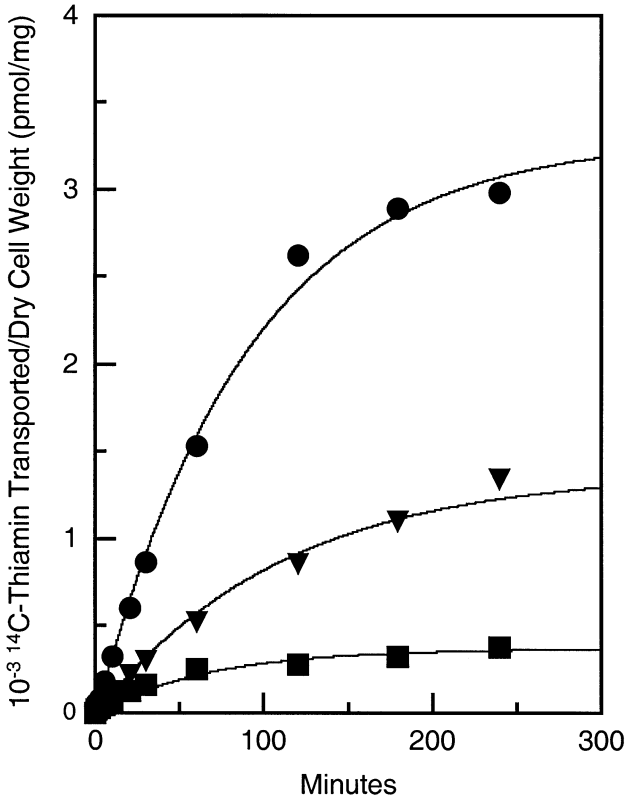

Fig. 1. Time course for 3 μM [*thiazole*-2-^{14}C]thiamin (24 Ci mol^{-1}) transport into 10 ml of intact *E. coli* KG33 (10^8 cells ml^{-1}) after no treatment (circles), treatment with 0.1 mM NEM (triangles), or treatment with 0.5 mM NEM (squares) in M9 minimal medium (supplemented with 0.2% glucose) at 37 °C (see text). The lines are drawn for a computer generated best fit to the data for an approach to an equilibrium intracellular concentration of thiamin with an observed first-order rate constant of 1.9×10^{-4} s^{-1}.

0.2% glucose) at 37 °C is shown in Fig. 2. Thiamin (2.5–1000 nM) accumulation into untreated *E. coli* KG33 exhibits Michaelis–Menten kinetics with values of $K_M = 15.2 \pm 1.7$ nM and $V_{max} = 46.1 \pm 2.9$ U mg^{-1}, as determined by a computer generated best fit of the data to both a Michaelis–Menten plot according to Eq. (1) (Fig. 2A, solid circles), and a computer generated best fit of the data to a Lineweaver–Burk reciprocal plot according to Eq. (2) (Fig. 2B, solid circles).

$$\nu = \frac{V_{max}[\text{thiamin}]}{[\text{thiamin}] + K_M} \quad (1)$$

$$\frac{1}{\nu} = \frac{K_M}{V_{max}}\left(\frac{1}{[\text{thiamin}]}\right) + \frac{1}{V_{max}} \quad (2)$$

Treatment of *E. coli* KG33 with 0.1–1.0 mM NEM resulted in an incremental decrease in V_{max} with no significant effect

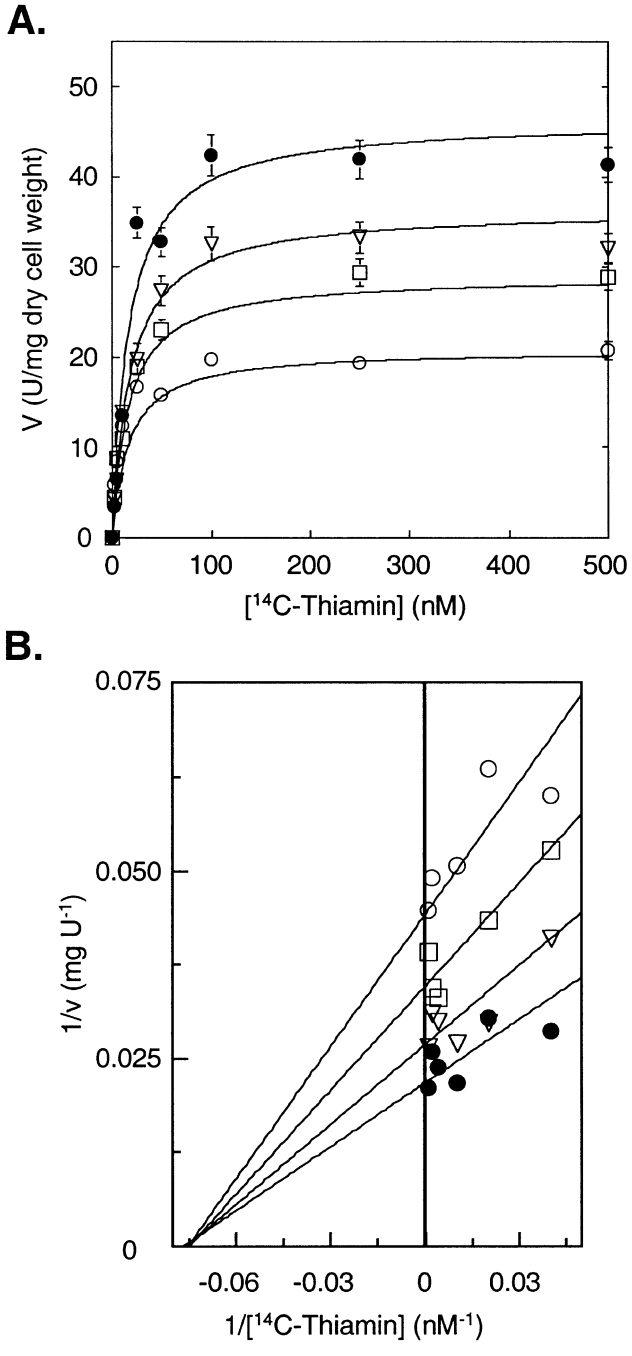

Fig. 2. Dependence on [thiamin] (2.5–1000 nM) of the initial rate for [*thiazole*-2-^{14}C]thiamin (24 Ci mol^{-1}) transport into 10 ml of intact *E. coli* KG33 (10^8 cells ml^{-1}) after no treatment (closed symbols) or treatment (open symbols) with 0.1–1.0 mM NEM in M9 minimal medium (supplemented with 0.2% glucose) at 37 °C. The lines are drawn for a computer generated best fit to the data for a Michaelis–Menten kinetic model according to Eq. (1) (A), or a computer generated best fit to the data for a Lineweaver–Burk reciprocal plot according to Eq. (2) (B) exhibiting inhibition by NEM with $K_i = 0.8$ mM. The initial rate values from the entire range of thiamin concentrations (2.5–1000 nM) were used for the computer generated best fit of the data to the Lineweaver–Burk reciprocal plot (panel B). However, we have expanded the region of the Lineweaver–Burk plot closest to the *y*-axis in order to better illustrate the apparent noncompetitive nature of the inhibition of thiamin transport by NEM. The Michaelis–Menten constants are as follows: $K_M = 15.2 \pm 1.7$ nM and $V_{max} = 46.1 \pm 2.9$ U mg^{-1} without NEM treatment (closed circles); $K_M = 15.8 \pm 1.5$ nM and $V_{max} = 36.9 \pm 1.7$ U mg^{-1} after treatment with 0.10 mM NEM (open triangles); $K_M = 15.0 \pm 1.8$ nM and $V_{max} = 28.8 \pm 1.1$ U mg^{-1} after treatment with 0.50 mM NEM (open squares); and $K_M = 15.1 \pm 1.9$ nM and $V_{max} = 21.7 \pm 0.9$ U mg^{-1} after treatment with 1.0 mM NEM (open circles).

on $K_M = 15.1 \pm 1.9$ nM (Fig. 2 and Table 1). The observed effect of NEM on the initial rate of thiamin accumulation is consistent with an apparent noncompetitive inhibition (simple intersecting linear noncompetitive inhibition) with a $K_i = 0.8 \pm 0.1$ mM. A classical noncompetitive inhibitor has no effect on substrate binding, and vice versa. The inhibitor and the substrate bind reversibly, randomly, and independently at different sites. However, a substance that can irreversibly inactivate an enzyme, such as NEM [25], is sometimes (incorrectly) called a noncompetitive inhibitor because V_{max} is decreased with no apparent effect on K_M. Therefore, our results are consistent with an irreversible inactivation of thiamin transport through a covalent modification of a cysteine(s) that is either critical for the transport complex or is present in a domain that is important for the transport of thiamin.

The observed accumulation of thiamin within the cells results from a combination of the net thiamin transported and the net thiamin exported. Although the transport of thiamin is an active process and little export is expected to occur, we wanted to confirm that the observed decrease in the accumulation of thiamin upon the addition of NEM results from an inhibition of the transport of thiamin and not an increase in the export of thiamin from the cells. Fig. 3 shows that there is little or no export ($k_{obsd} \leq 5.0 \times 10^{-6}$ s^{-1}) of [*thiazole*-2-^{14}C]thiamin from NEM-treated or untreated *E. coli* KG33 in M9 minimal medium at 37 °C. NEM (≤ 1.0 mM) was shown not to cause detectable loss of intracellular [*thiazole*-2-^{14}C]thiamin upon treatment of preloaded cells and NEM treatment before preloading the cells with thiamin had no effect on export (data not shown).

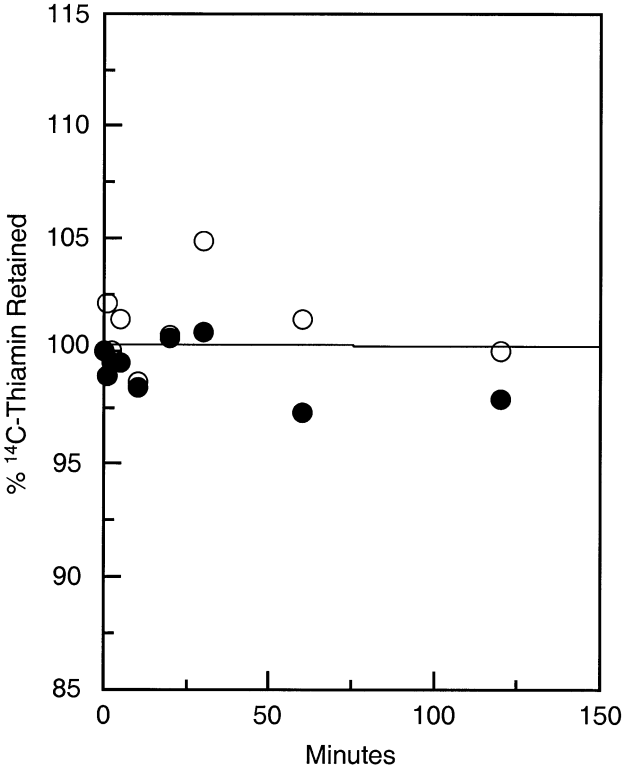

Fig. 3. Time course for [*thiazole*-2-^{14}C]thiamin export from 10 ml of untreated intact *E. coli* KG33 (10^9 cells ml^{-1}) (closed symbols) or cells treated with 1.0 mM NEM (open symbols) at 37 °C in M9 minimal medium containing 4% glucose. The line is drawn for an observed first-order rate constant of $\leq 5.0 \times 10^{-6}$ s^{-1}.

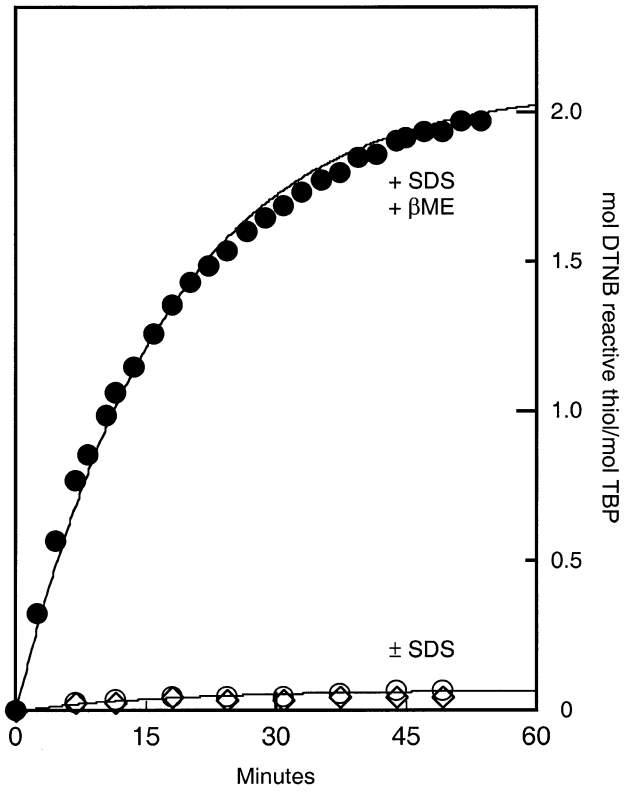

Fig. 4. DTNB reaction with native (open symbols) and reduced (solid symbols) plasmid-derived *E. coli* periplasmic TBP in the presence (circles) and absence (diamonds) of 0.2% SDS. TBP (270 μg; 8 nmol) was incubated at 25 °C in 50 mM aqueous sodium phosphate (pH 7.5) buffer containing 5 mM sodium chloride and 350 μM DTNB in the presence and absence of 0.2% SDS. For the determination of the total thiol content of TBP ("+SDS, +βME" curve), the protein was incubated with 0.2% SDS and 250 mM βME at 37 °C for 30 min before initiation of the reaction with DTNB (see text). The line for the "+SDS, +βME" curve is drawn for a first-order rate constant of $(8.7 \pm 0.1) \times 10^{-4}$ s^{-1}.

Finally, the transport of thiamin involves a periplasmic space binding protein, which acts as the initial receptor in the transport process. We have recently cloned and purified the TBP, which contains only two cysteines, to apparent homogeneity [13]. Therefore, we wished to determine if the cysteines in TBP as isolated from the cells were capable of being modified by sulfhydryl-specific reagents. The time course for reaction of TBP with the sulfhydryl-specific reagent DTNB at pH 7.5 and 25 °C in the presence and absence of SDS is shown in Fig. 4. There was no detectable reaction of TBP with DTNB in the presence and absence of SDS. With denatured and reduced TBP, two thiol groups react by a single rate process with a second-order rate constant of approximately 2.5 M^{-1} s^{-1} [=$(8.7 \pm 0.1) \times 10-4$ s^{-1}/ 3.5×10^{-4} M DTNB]. This result demonstrates that the

two thiols present in TBP, as isolated from the cells, exist in an oxidized state and are unable to react with sulfhydryl-specific reagents such as NEM.

4. Discussion

Thiamin transport in *E. coli* occurs by a periplasmic permease belonging to the ABC transporter family. It was demonstrated that the sulfhydryl-specific reagent NEM inhibits the transport of thiamin [16], suggesting that the modification of a thiol alters a domain that is critical for thiamin accumulation. In the present work, we performed a mechanistic study on the inhibitory effect of NEM to better understand the mechanism of thiamin transport. We have demonstrated that in the presence or absence of NEM, the accumulation of thiamin in *E. coli* occurs by an active transport process and follows Michaelis–Menten kinetics. Incubation with NEM exhibits an apparent noncompetitive inhibition of thiamin transport, which is consistent with the irreversible inactivation of a component of the transport complex [25]. Since it has been previously demonstrated that the periplasmic space TBP is insensitive to modification by NEM [11], we therefore conclude that the inhibition of NEM must occur at either the membrane-bound component (thiP) or the ATPase subunit (thiQ).

The fact that thiamin accumulation follows Michaelis–Menten kinetics, either in the presence or absence of NEM (Fig. 2), allows several constraints to be placed on the mechanism of thiamin transport according to the analyses of Krupka [8] and Bohl and Boos [9]. First, in the absence of thiamin, the binding protein will exist in the "open" conformation (the substrate binding site is accessible to solvent). Second, the rate-limiting step for transport is the binding protein-mediated transfer of thiamin to the integral membrane-bound carrier protein. Finally, the rate of the carrier protein conformational change, resulting in the translocation of thiamin into the cytoplasm, is fast compared to the release of the thiamin from the carrier protein.

We also observed an apparent first-order accumulation of thiamin, either in the presence or absence of NEM (Fig. 1). The observed first-order rate constant for the accumulation of the substrate (k_{obsd}) is equal to the sum of the rate constant for uptake (k_{in}) and the rate constant for export (k_{out}) as described by Eq. (3).

$$k_{obsd} = k_{in} + k_{out} \quad (3)$$

The absence of detectable export of thiamin, TMP, or TDP from *E. coli* KG33 (Fig. 3) establishes an upper limit of $k_{out} \leq 5.0 \times 10^{-6}\ s^{-1}$ in thiamin accumulation. Because the export of thiamin is negligible, we can therefore conclude that $k_{obsd} \approx k_{in}$, where k_{in} is a function of all the rate constants for each step of the transport process. In addition, V_{max} for thiamin transport is directly proportional to the observed first-order rate constant for uptake (k_{in}) and the total concentration of active thiamin periplasmic permease (E_T) as given in Eq. (4) [26].

$$V_{max} = k_{in}[E_T] \quad (4)$$

Because NEM decreases the value of V_{max} while k_{obsd} ($\approx k_{in}$) is independent of [NEM] (Table 1), the observed decrease in V_{max} for thiamin transport must be attributed to an apparent decrease in the total concentration of active transporter [E_T]. This conclusion is further supported by the apparent noncompetitive inhibition of thiamin transport by NEM (Fig. 2). Reagents that modify a functional group or a domain of an enzyme that is essential for maintaining the catalytically active conformation of the enzyme commonly exhibit apparent noncompetitive inhibition. Some enzymes possessing an essential thiol(s) are inhibited noncompetitively by thiol-specific reagents, such as NEM, suggesting that such thiol groups must be intact for the enzyme to retain the active conformation or for proper protein–protein interactions to be achieved [27]. The net effect of the inhibitor is to make it appear as if less total enzyme, or in the case of the present study, the active transporter, is present [26].

The decrease in the total concentration of active thiamin periplasmic permease could in principle result from the covalent modification of any of the components of the transporter including the periplasmic space binding protein TBP, the integral membrane-bound component thiP, or the ATPase subunit thiQ. Inhibition of thiamin transport by NEM could therefore occur by any of several different mechanisms: (i) the inhibition of the binding of thiamin by TBP; (ii) the inhibition of the interaction between liganded TBP and the membrane-bound carrier protein thiP and subsequent transfer of thiamin from TBP to thiP; (iii) the inhibition of the ATPase activity of thiQ; or (iv) the inhibition of the interaction of thiP with the ATPase subunit thiQ and subsequent translocation of thiamin across the membrane. Modification of an essential thiol(s) required for the binding of thiamin by TBP cannot explain a decrease in active transporter for two reasons. First, the value of K_M for thiamin transport under conditions where K_M of transport approaches the K_D of the binding protein [9] is independent

Table 1
Steady-state kinetic parameters for thiamin transport into *E. coli* KG33 after treatment with NEM[a]

[NEM] (mM)	$10^4\ k_{obsd}$ (s^{-1})	K_M (nM)	V_{max} (U mg^{-1})[b]
0	1.8 ± 0.2	15.2 ± 1.7	46.1 ± 2.9
0.10	1.5 ± 0.2	15.8 ± 1.5	36.9 ± 1.7
0.50	2.5 ± 0.5	15.0 ± 1.8	28.8 ± 1.1
1.0	N.D.	15.1 ± 1.9	21.7 ± 0.9

Abbreviations: NEM, *N*-ethylmaleimide; N.D., not determined.

[a] At 37 °C in M9 minimal medium supplemented with 0.2% glucose containing 3 μM [*thiazole*-2-^{14}C]thiamin (24 Ci mol^{-1}) at a density of 10^8 cells ml^{-1}.

[b] An activity unit (U) is defined as one picomole of thiamin transported per minute at 37 °C and is normalized for dry cell weight (mg).

of [NEM] (Table 1). Second, and more directly, NEM does not inhibit thiamin binding to TBP in vitro [11]. Taken together, these results support the conclusion that thiamin binding by TBP in vivo is unaffected by NEM.

Our data also indicate that inhibition does not occur at the level of the interaction between liganded TBP and thiP or the transfer of thiamin to thiP. As described above, the observed rate constant (k_{obsd}) is a function of all steps of the transport process, which includes binding of thiamin by TBP, transfer of thiamin from TBP to thiP, ATP hydrolysis, and release of thiamin into the cytoplasm [8]. However, the binding of thiamin by TBP [13] and ATP hydrolysis by the ATPase subunit [17,20,28] are rapid processes relative to the rate-limiting step. In addition, the constraints imposed by Michaelis–Menten kinetics require that release of thiamin into the cytoplasm is also rapid (see above). The consequence of the rapid nature of these steps is that the k_{obsd} for thiamin transport is essentially equivalent to the rate constant for the rate-limiting step, which is the interaction of liganded TBP with thiP and the transfer of thiamin from TBP to thiP [8]. Because k_{obsd}, and therefore the rate-limiting step, are unaffected by NEM, we conclude that inhibition of thiamin transport by NEM does not occur at the level of the interaction of liganded TBP with thiP or the transfer of thiamin from TBP to thiP and must therefore occur at the level of the integral membrane-bound component or the ATPase subunit.

NEM was reported to inhibit the actions of a variety of ABC transporters by covalently modifying a cysteine that is present in the Walker A motif of the ATPase subunit, the region responsible for the binding of ATP, and thereby inhibiting the binding of ATP [17–20,29,30]. An analysis of the primary amino acid sequence of thiQ, the ATPase subunit of the thiamin transporter, revealed that no cysteines are present in its Walker A motif (Fig. 5A). Therefore, NEM would be unable to inhibit the binding or hydrolysis of ATP by thiQ. However, a primary amino acid sequence analysis of thiQ and of the integral membrane component thiP revealed that only three cysteines within these proteins are predicted to be accessible to modification by NEM. One of the cysteines in thiP is present in a loop structure that our primary amino acid analysis predicted to be in the periplasmic space. However, our mechanistic analysis is inconsistent with modification and subsequent inhibition occurring in the periplasmic space (see above). The two remaining cysteines are present in the ABC signature domain of thiQ [3] (Fig. 5B) and the "EAA loop" of thiP [31] (Fig. 5C). The ABC signature and the EAA loop are believed to be important for the interaction between the integral membrane component and the ATPase subunit [4,30]. Therefore, it is conceivable that modification of either of these proteins by NEM could disrupt the interaction between thiQ and thiP and thereby inhibit thiamin transport.

In summary, we have performed a kinetic study in order to elucidate the mechanism of inhibition of thiamin transport by NEM. Through a kinetic analysis, combined with an analysis of the primary amino acid sequences of the integral membrane component thiQ and the ATPase subunit thiP, our data supports the conclusion that NEM inhibits thiamin transport by covalently modifying one or both of the cysteines present in the domains responsible for mediating the interaction between thiQ and thiP. This mechanism of inhibition is different from that demonstrated for other ABC transporters in which the modification of a critical cysteine by NEM inhibits the binding of ATP by the ATPase subunit. Instead, our results support the mechanism in which thiamin accumulation is inhibited because ATP hydrolysis in uncoupled from the transport process.

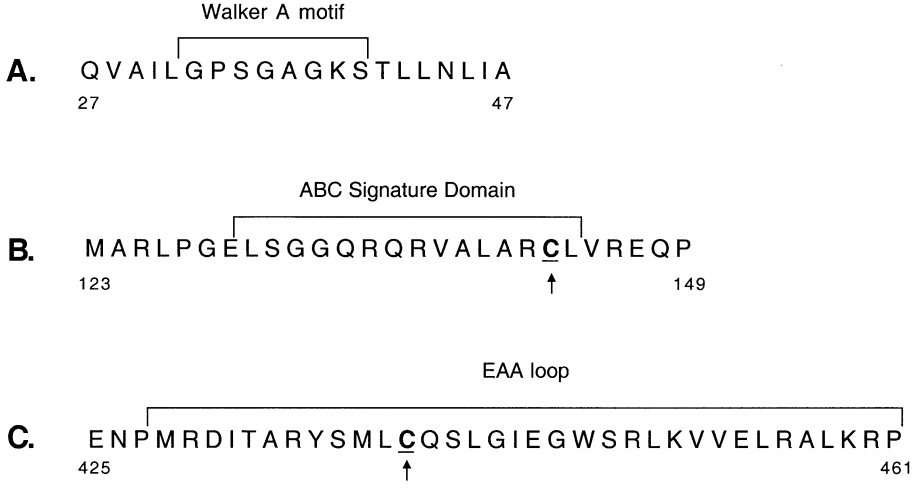

Fig. 5. Structural location of NEM accessible cysteines in the ATPase subunit and the integral membrane component of the thiamin ABC transporter. The key domains are indicated above the sequence and the amino locations of these domains are indicated below the sequence. The cysteines are in bold, underlined, and indicated with an arrow.

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