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Demonstration of dimer formation of the cytoplasmic domain of a transmembrane osmosensor protein, EnvZ, of *Escherichia coli* using Ni-histidine tag affinity chromatography

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Abstract **EnvZ is a transmembrane osmosensor which regulates the phosphorylation of OmpR, a transcription factor for *ompF* and *ompC* genes which encode the major outer membrane porin proteins, OmpF and OmpC in *Escherichia coli*. Autophosphorylation of EnvZ occurs through a transphosphorylation reaction between two EnvZ molecules. To elucidate the molecular mechanism of signal transduction by EnvZ, we examined the dimer formation of the EnvZ cytoplasmic domain [EnvZ(C)]. For this purpose, we developed a method to determine the complex formation between the purified EnvZ(C) and the purified His6-EnvZ(C) by means of Ni-6xhistidine tag affinity chromatography. When the mixture of EnvZ(C) and His6-EnvZ(C) was applied to Ni-NTA resin, both His6-EnvZ(C) and EnvZ(C) were bound to the resin, indicating that EnvZ can form an oligomer without the periplasmic and transmembrane domains. Binding experiments using the Ni-NTA resin revealed that EnvZ(C) forms a dimer with the K_a value for dimerization being approximately 10^5 M^{-1} in the equilibrium state.**

Key words: Dimer formation; EnvZ; Histidine kinase; OmpR; Osmosensor

1. Introduction

EnvZ is a transmembrane protein in the cytoplasmic membrane which functions as an osmosensor in *Escherichia coli* to regulate the production of two major outer membrane porin proteins, OmpF and OmpC [1–3]. EnvZ is an autokinase that becomes phosphorylated at histidine-243 by ATP through a transphosphorylation reaction between EnvZ molecules, and thus is called a histidine kinase [4]. The phosphate group is subsequently transferred to OmpR, which activates this transcription factor to allow regulation of the *ompF* and *ompC* genes [5,6]. In addition to its autokinase and phosphotransferase activities, EnvZ can also act as a phosphatase which dephosphorylates phosphorylated OmpR, and thus inactivates OmpR [7]. It has been demonstrated that the ratio of kinase and phosphatase activities is modulated in response to the osmolarity of the medium and plays an important role in the osmoregulation of *ompF* and *ompC* [8,9].

EnvZ consists of three domains: a periplasmic sensor domain, transmembrane domains, and a cytoplasmic catalytic domain [10]. Although the periplasmic domain is considered to function as an osmotic sensor, its natural ligand has not been identified. Mutational analyses of the transmembrane domains suggest that an intimate intermolecular interaction between the two membrane-spanning segments is crucial for transmembrane signaling in response to external osmotic signals [11]. The signal thus transduced across the membrane regulates the enzymatic activities of the cytoplasmic domain to control the level of phosphorylation of OmpR. Using a hybrid protein, Taz1, consisting of the receptor domain of Tar protein, an aspartate chemoreceptor, and the cytoplasmic catalytic domain of EnvZ [12], it has been demonstrated that dimer (oligomer) formation is necessary for signal transduction of EnvZ [13,14]. The dimer or oligomer formation of receptor proteins such as an epidermal growth factor (EGF) receptor and a human growth factor (hGF) receptor has been shown to play an important role for transmembrane signaling of membrane bound receptors. The EGF receptor [15] and the hGF receptor [16] form dimers in a ligand-dependent manner, while Tar protein exists as a dimer in a ligand-independent manner [13,14]. It is not yet clear whether the oligomerization of EnvZ is ligand-dependent or not. In addition, the number of subunits in the EnvZ oligomer and important regions for the oligomerization of the EnvZ molecule have not yet been determined in detail.

In the present report, we unambiguously demonstrate the dimer formation of the cytoplasmic domain of EnvZ [EnvZ(C)] by developing Ni-histidine tag affinity chromatography. The K_a value for the dimer formation was estimated to be 10^5 M^{-1}, which is similar to the K_a value for the dimer formation of the cytoplasmic domain of the aspartate receptor, Tar [17,18]. The present results provide insight into the structure and function of the cytoplasmic signaling domain of EnvZ.

2. Materials and methods

2.1. Construction of plasmids for expression of EnvZ(C) and His6-EnvZ(C)

To express the cytoplasmic domain of EnvZ under a T7 promoter, plasmid pET11a-Z(C) was constructed as follows. The unique *Eco*RI site of pET11a plasmid (Novagen, Madison, WI, USA) was first eliminated by *Eco*RI digestion, followed by Klenow treatment, and ligation. The oligonucleotides (sense, TACGAATTCCGGCCA, and antisense, GCTTAAGGCCGGTAT) which contained an *Eco*RI site were inserted into the unique *Nde*I site of the pET11a-derivative described above. The 1.5-kb *Eco*RI fragment corresponding to the cytoplasmic domain of EnvZ(C) (residues 180–450) was isolated from the *ompC:envZ*^{c} plasmid [19] into the newly created *Eco*RI site to construct pET11a-Z(C). The EnvZ(C) protein thus produced contains

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Abbreviations: Ni-NTA, Ni^{2+}-nitrilotriacetic acid agarose; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate-acrylamide gel electrophoresis

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4 extra amino acid residues (Met-Ala-Gly-Ile) at the amino terminal end consisting of a total of 271 residues.

Plasmid pPH001 for the production of histidine-tagged EnvZ(C) was constructed as follows. The first PCR fragment was made using primer 1 (5′-TAATACGACTCACTAT-3′) and primer 2 (5′-CTGGATACGAATTCC(ATG)$_6$GCC-3′) resulting in a His6 tag. The second PCR fragment was made using primer 3 (5′-GGAATTCGTATCCAG-3′) and primer 4 (5′-GCGCAAGTCGT-GACTTACCCC-3′). Since the first 15 nucleotides of primers 2 and 3 are complementary, the third PCR fragment was constructed with primer 1 and primer 4 using the first and second PCR fragments as a template. The third PCR fragment carrying His6-EnvZ(C) was digested with *Xba*I-*Nde*I, and the 300-bp *Xba*I-*Nde*I fragment was inserted into the *Xba*I-*Nde*I digested pET11a-Z(C) to construct pPH001. The product from this plasmid contained 9 extra residues [Met-Gly-(His)6-Gly-EnvZ(C)] at the amino-terminal end of the C-terminal fragment of EnvZ from residue 180 to 450. The product was designed as His6-EnvZ(C) and was confirmed by DNA sequencing (Sequenase).

2.2. Purification of His6-EnvZ(C) and EnvZ(C)

E. coli BL21(DE3) [20] transformed with pPH001 was grown at 37°C in 2 l of M9 medium containing casamino acid (20 g/l) supplemented with 50 μg/ml ampicillin. The expression of His6-EnvZ(C) was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at mid log phase. After a 3 h incubation, cells were harvested, and washed with buffer A (50 mM sodium phosphate buffer (pH 7.8) containing 0.3 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Cells were resuspended in 20 ml of buffer A, sonicated on ice, and treated with ribonuclease A (10 μg/ml) and deoxynuclease (5 μg/ml) at 4°C for 20 min. Unbroken cells were removed by centrifugation (4000×*g* for 20 min), and the supernatant was again centrifuged at 200 000×*g* for 20 min to remove the insoluble membrane fractions. The supernatant thus obtained was mixed with 4 ml Ni-NTA resin [21], previously equilibrated with buffer A. Ni affinity chromatography was performed with a Bio-Rad Econo system. After washing the Ni-NTA resin with buffer B (50 mM sodium phosphate buffer (pH 6.0) containing 0.3 M NaCl, 10% glycerol, and 1 mM PMSF), proteins were eluted with a linear gradient of 0–0.2 M imidazole in buffer B and the fractions were analyzed on SDS-PAGE [22]. Fractions from 0.1 to 0.2 M were pooled and dialyzed against buffer C (20 mM Tris-HCl (pH 7.4) containing 0.3 M NaCl, 20% glycerol, 10 mM β-mercaptoethanol, and 1 mM PMSF) before use. EnvZ(C) was purified with anion exchange chromatography (DE52) as reported previously [19]. Protein concentrations of the purified His6-EnvZ(C) and EnvZ(C) were determined using the Bio-Rad protein assay method.

2.3. Biochemical activities of His6-EnvZ(C)

The autophosphorylation of His6-EnvZ(C) with [γ-^{32}P]ATP, phosphorylation of OmpR and dephosphorylation of phosphorylated OmpR by His6-EnvZ(C) were carried out as described previously [13,23].

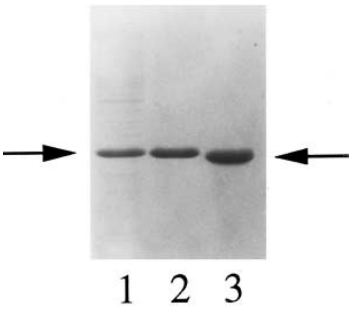

Fig. 1. Purification of His6-EnvZ(C) and EnvZ(C). Lane 1, total cellular proteins from *E. coli* harboring pPH0001 grown in the presence of 0.5 mM IPTG; lane 2, His6-EnvZ(C) purified by Ni-NTA affinity chromatography; lane 3, purified EnvZ(C). An arrow at the left side shows the position of His6-EnvZ(C) and an arrow at the right side the position of EnvZ(C). Proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue.

2.4. Binding assay

The binding experiment between His6-EnvZ(C) and EnvZ(C) was carried out as follows. The purified His6-EnvZ(C) and EnvZ(C) were mixed in buffer C at several different concentrations and incubated at 25°C for 30 min. For the determination of the K_a value, equimolar concentrations of His6-EnvZ(C) and EnvZ(C) were mixed and incubated for 60 min. After incubation, Ni-NTA resin (5 μl resin/5 μl; buffer C) was added to the reaction mixture and the mixture was incubated on ice for 30 min with occasional shaking. The Ni-NTA resin was removed to an ultrafree C3VV filter (Millipore, MA) and filtrated at 4. The resulting Ni-NTA resin was washed three times with buffer C at 4°C within 3 min to avoid the dissociation of the complex. The Ni-NTA resin was treated either with 10 μl of 8 M urea or with 10 μl of 50 mM EDTA at 25°C for 5 min, and then filtrated. This step for protein elution was repeated twice and the eluates were combined. The eluates were applied to SDS-PAGE. The gels were stained with Coomassie brilliant blue and the amounts of proteins were estimated using the Bio-Rad Model GS-670 Imaging Densitometer.

3. Results

3.1. Expression and purification of the cytoplasmic domain of EnvZ

EnvZ is a member of a histidine kinase family which share common amino acid sequence motifs: H, G1, and G2 boxes [24]. The C-terminal cytoplasmic domain of EnvZ [EnvZ(C)] from residue 180 to 450 contains all of these histidine kinase motifs. In His6-EnvZ(C), an extra 9 amino acid residues (Met-Gly-His6-Gly) was added to the amino terminal end of EnvZ(C). Both EnvZ(C) and His6-EnvZ(C) were expressed as soluble proteins in *E. coli* and retained both kinase and phosphatase activities. Fig. 1 shows an SDS-PAGE pattern of purified EnvZ(C) (lane 3) and His6-EnvZ(C) (lane 2). It is important to note that these proteins can be separated on SDS-PAGE because of the difference in molecular weights (approximately 1 kDa). As previously shown for EnvZ(C) [25], purified His6-EnvZ(C) can be autophosphorylated with [γ-^{32}P]ATP (lane 1, Fig. 2), can phosphorylate OmpR (lanes 2–4, Fig. 2), and can also dephosphorylate phosphorylated OmpR (lanes 5–7, Fig. 2). The specific activities of His6-EnvZ(C) for those activities were comparable with those of EnvZ(C) (data not shown), indicating that the histidine tag did not affect the function of EnvZ(C).

3.2. Detection of the EnvZ(C) dimer using Ni-histidine affinity

Assuming that the cytoplasmic domain of EnvZ can form a dimer, three different dimers are expected to form in the mixture of His6-EnvZ(C) and EnvZ(C) as depicted in the upper part of Fig. 3. Of these dimers, His6-EnvZ(C) homodimer and His6-EnvZ(C)-EnvZ(C) heterodimers bind to Ni-NTA resin, while EnvZ(C) homodimers do not bind (see Fig. 3). When the resin is washed with a denaturant solution containing 8 M urea solution, only EnvZ(C) is expected to be released from the resin as shown in the lower part of Fig. 3. Alternatively, if the resin is treated with EDTA, both His6-EnvZ(C) and EnvZ(C) are eluted.

Fig. 4 shows such an experiment analyzing eluted proteins on SDS-PAGE. In this experiment, the total protein concentrations were kept constant in order to estimate oligomer formation as mentioned below. Equimolar concentrations of His6-EnvZ(C) and EnvZ(C) (lanes 2 and 5) were mixed and incubated for 30 min, followed by the addition of Ni-NTA resin to the mixture. As shown in lane 2, only EnvZ(C) was eluted with 8 M urea, and importantly when only EnvZ(C) was mixed with Ni-NTA resin, it did not significantly bind to

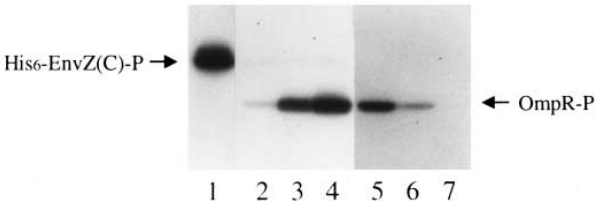

Fig. 2. In vitro biochemical activities of the purified His6-EnvZ(C). Autophosphorylation activity (lane 1), purified His6-EnvZ(C) (1 μg) was mixed with 1 μCi of [γ-^{32}P]ATP in 15 μl of 50 mM HEPES buffer (pH 8.0) containing 5 mM $CaCl_2$ and 50 mM KCl, and incubated for 5 min at 25°C. The reaction was stopped by the addition of SDS-PAGE sample buffer. Phospho-transfer activity (lanes 2–4), purified His6-EnvZ(C) (1 μg) was mixed with OmpR (1 μg) at 25°C. After adding 1 μCi of [γ-^{32}P]ATP, the reaction was stopped at 15 s, 2 or 5 min (lanes 2, 3, and 4, respectively). For phosphatase activity (lanes 5–7), OmpR was first phosphorylated with the membrane fraction containing EnvZ(T247R) phosphorylated with 10 μCi of [γ-^{32}P]ATP as described previously [13,23]. After removing the membrane fractions by centrifuge, phosphorylated OmpR (1 μg) was incubated with 1 mM ADP and His6-EnvZ(C) (1 μg) at 25°C. The reaction was stopped at 15 s, 3 or 10 min (lanes 5, 6, and 7, respectively).

the resin (lane 3). Non-specific binding of EnvZ(C) to the resin was less than 10% of the specific binding of EnvZ(C) mediated by His6-EnvZ(C) as determined by densitometric analysis. When the resin was washed with 50 mM EDTA, His6-EnvZ(C) was released from the resin as shown in lane 4 (His6-EnvZ(C) alone) and lane 5 (His6-EnvZ(C) and EnvZ(C)). The fact that EnvZ(C) eluted together with His6-EnvZ(C) (lane 5) demonstrated that EnvZ(C) binding to the resin was mediated by His6-EnvZ(C).

To determine the number of subunits in the complex that formed on Ni-NTA resin, binding experiments described in Fig. 3 were performed at several concentrations. The proteins were eluted from Ni-NTA resin by 50 mM EDTA, and subjected to SDS-PAGE. The density of protein band stained with Coomassie brilliant blue was determined by the Bio-Rad Model GS-670 Imaging Densitometer. When the concentrations of both proteins were kept at 3×10^{-4}, 1×10^{-4}, and 6×10^{-5} M, the ratios of EnvZ(C) to His6-EnvZ(C) bound on Ni-NTA resin were 0.42, 0.42, and 0.41, respectively. These data suggest that the oligomer formation was almost completed at the range of concentrations used because the ratio was not concentration-dependent. The theoretical ratio for the dimer formation of EnvZ(C) and His6-EnvZ(C) at saturation is 0.5 when equimolar concentrations of His6-EnvZ(C) and EnvZ(C) were mixed (see Fig. 3); in the case of the trimer, the theoretical ratio is 0.75. While the experimental value (0.42) was slightly lower (84%) than 0.5 for the dimer formation, this value is consistent with a dimer formation. Therefore, one can conclude that under the conditions used, the cytoplasmic domain of EnvZ forms a dimer.

3.3. Determination of apparent Ka value of EnvZ(C) dimer formation

Subsequent binding experiments were carried out over a wider range of protein concentrations between 10^{-7}–10^{-4} M as shown in Fig. 5. In these experiments, equimolar concentrations of His6-EnvZ(C) and EnvZ(C) were used. The mixtures were incubated in Tris-HCl buffer (pH 7.4) at 25°C for 60 min before Ni-NTA resin was added. Proteins bound to the resin were eluted with 50 mM EDTA, and their amounts were estimated by densitometer analyses after SDS-PAGE. Data analyses were performed as follows. According to the monomer-dimer equilibrium, the total protein concentration is presented by Eq. 1, and the K_a value for the dimer formation is presented by Eq. 2, where $[M_0]$ represents a total protein concentration, [M] a monomer concentration, [M2] a dimer concentration, and K_a is an association constant. The ratio $[M]/[M_0]$ is a function of the association constant (K_a) for the dimer formation and is given as Eq. 3.

$$[M_0] = [M] + 2[M2] \quad (1)$$

$$K_a = [M2]/[M]2 \quad (2)$$

$$[M]/[M_0] = \{-1 + (1 + 8K_a[M_0])^{1/2}\}/4K_a[M_0] \quad (3)$$

As mentioned, we estimated the ratios of densities of EnvZ(C) to those of His6-EnvZ(C) on SDS-PAGE. These values were divided by 0.5 to obtain $2[M2]/[M_0]$ because the ratio of EnvZ(C) to His6-EnvZ(C) is 0.5 when all protein molecules form dimers. The ratios of monomers to total protein ($[M]/[M_0]$) were calculated from the ratios ($2[M2]/[M_0]$) mentioned above using Eq. 1. In Fig. 5, two theoretical curves generated by Eq. 3 are also shown for $K_a=10^6$ M^{-1} and 10^5 M^{-1}. The experimental curve fits well with that of $K_a=10^5$ M^{-1}, indicating that the K_a value in equilibrium for the dimer formation of the cytoplasmic domain of EnvZ is approximately 10^5 M^{-1}.

4. Discussion

The results presented here clearly demonstrate that the cy-

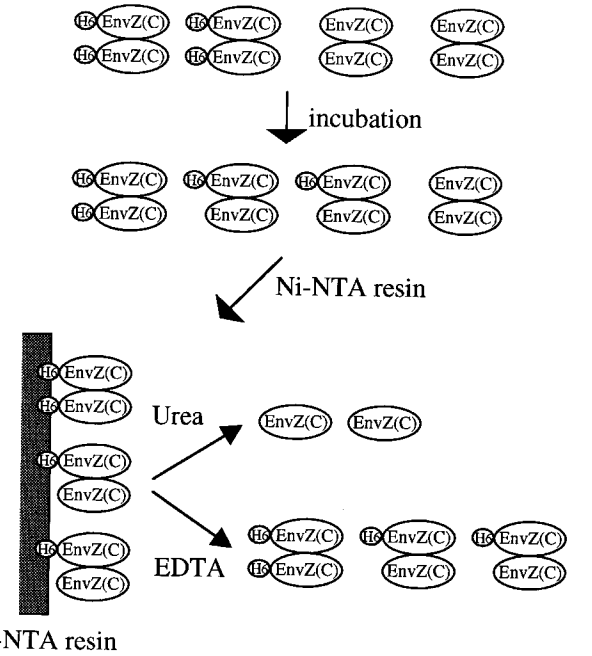

Fig. 3. Strategy for the determination of the subunit composition of EnvZ(C). This figure illustrates a theoretical situation in which His6-EnvZ(C) and EnvZ(C) are assumed to be mixed in equimolar concentrations enough to form dimers completely. Only molecular species containing the His tag can be trapped on Ni-NTA resin. See text for details. H6 represents six histidine residues added at the N-terminal end of EnvZ(C). Note that monomers existing in the system for the formation of dimers are not shown in the figure.

toplasmic domain of EnvZ forms a dimer and participates in monomer-dimer equilibrium. Prior to this experiment, we employed the size exclusion chromatography to determine the oligomer formation of EnvZ(C) (data not shown). EnvZ(C) was eluted at the dimer position which was estimated from retention times of standard globular proteins on the column. However, it appeared to be a monomer protein because it did not show the monomer-dimer equilibrium on the column after dilution of the protein solution. The monomer EnvZ(C) might have a non-globular molecular shape like a cytoplasmic domain of Tar protein [17,18]. Therefore, in order to estimate the K_a value for the dimer formation of EnvZ(C), we developed a binding assay system to determine the oligomer formation using the Ni-histidine tag affinity. When EnvZ(C) was added to Ni-NTA resin bound His6-EnvZ(C), the EnvZ(C) binding to the resin was not significant. Dimer formation was detected only when the mixture of EnvZ(C) and His6-EnvZ(C) was incubated before the addition of Ni-NTA resin. This suggests that once His6-EnvZ(C) is bound to Ni-NTA resin, it is unable to form a dimer, which is probably due to steric hindrances caused by the binding.

In this study, we used the purified EnvZ(C) and His6-EnvZ(C), which are supposed to be non-phosphorylated forms, to determine the dimer formation. Although it is difficult to accurately estimate the concentrations of phosphorylated and non-phosphorylated EnvZ(C), a preliminary experiment indicated that the phosphorylation of EnvZ(C) did not affect the dimer formation (data not shown). Using the present method, we were also able to demonstrate the binding of OmpR to EnvZ(C) (data not shown). However, an accurate K_a value could not be obtained because of its lower affinity to EnvZ(C). Interestingly, in the presence of ATP, OmpR binding to EnvZ(C) was weaker than in the absence of ATP (data not shown). Further study on the role of the dimerization for the kinase and phosphatase activity is in progress in order to investigate signal transduction of EnvZ.

The oligomerization of several proteins has been studied and characterized. For example, the cytoplasmic domain and periplasmic domain of Tar protein can form dimers, and the K_a values for the dimer formation are approximately

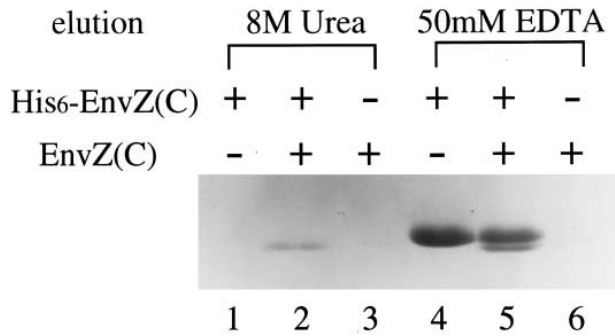

Fig. 4. Interaction between His6-EnvZ(C) and EnvZ(C). The interaction of EnvZ(C) with His6-EnvZ(C) was examined as shown in Fig. 3. Proteins bound to Ni-NTA resin were eluted with 8 M urea (lanes 1, 2, and 3) or 50 mM EDTA (lanes 4, 5, and 6). Lanes 1 and 4, only His6-EnvZ(C) (10^{-5} M) was treated with Ni-NTA resin; lanes 2 and 5, the mixture of His6-EnvZ(C) (5×10^{-6} M) and EnvZ(C) (5×10^{-6} M) was treated with Ni-NTA resin; lanes 3 and 6, only EnvZ(C) (10^{-5} M) was treated with Ni-NTA resin. The upper protein band corresponds to His6-EnvZ(C) and the lower band corresponds to EnvZ(C). Nonspecific binding of EnvZ(C) to Ni-NTA resin was less than 10% of the specific binding.

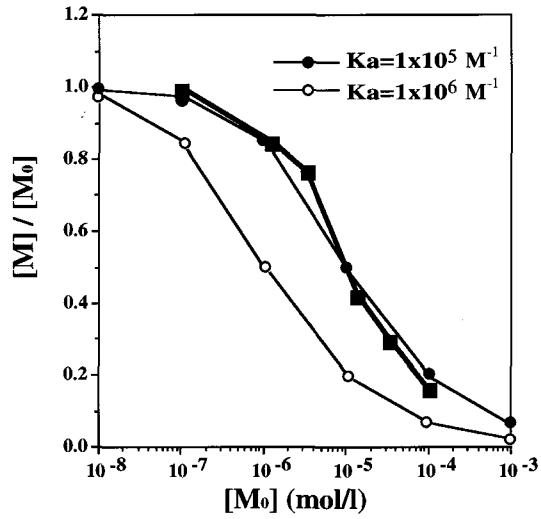

Fig. 5. Efficiencies of EnvZ(C) dimer formation. The [M]/[M_0] ratios were calculated from the densitometer analysis based on the densities of His6-EnvZ(C) and EnvZ(C) bands eluted with 50 mM EDTA on SDS-PAGE as described in the text. Filled squares represent experimental data, filled and open circles represent theoretical curves generated by Eq. 3 in the text for the K_a values of 10^5 and 10^6 M^{-1}, respectively. [M], monomer concentration; [M_0], total protein concentration. Experimental data were plotted as the average of two data sets. Nonspecific binding was less than 10% of the specific binding.

0.5×10^5 M^{-1} and 2×10^5–2×10^6 M^{-1}, respectively, as estimated from the K_d values [17,18,26]. Those K_a values are similar to the K_a value for dimer formation of His6-EnvZ(C) that was estimated to be 10^5 M^{-1}. It has been reported that EnvZ exists in *E. coli* at a very low level [27,28]. However, since the EnvZ molecules are exclusively localized in the inner membrane, their concentration may be enough to effectively form dimers. The K_a value for the dimer formation obtained in the present paper was carried out with the C-terminal fragment of EnvZ from residue 180 to 450, which does not contain the periplasmic domain and the transmembrane domains. Therefore, the dimerization of EnvZ occurs in a ligand-independent manner. On the basis of the three-dimensional structure of the Tar periplasmic domain [29], it is quite possible that the periplasmic domain of EnvZ also participates in the dimer formation. Similarly, the transmembrane domains are likely to be involved in the dimer formation. Therefore, the K_a value for the dimer formation of the entire EnvZ molecules in the membrane is probably much higher than 10^5 M^{-1}. In addition, one cannot exclude the possibility of the tetramer formation for entire EnvZ as shown in the Trg chemoreceptor [30].

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