EmrE is a small multidrug transporter, 110 amino acids long that extrudes various drugs in exchange with protons, thereby rendering *Escherichia coli* cells resistant to these compounds. Negative dominance studies and radiolabeled substrate-binding studies suggested that EmrE functions as an oligomer. Projection structure of two-dimensional crystals of the protein revealed an asymmetric dimer. To identify the functional unit of EmrE, a novel approach was developed. In this method, quantitative monomer swapping is induced in detergent-solubilized EmrE by exposure to 80 °C,300pxrów(289,253),(733,804)(289,253),(733,804)(289,253),(733,804)that does not impair transport activity. Oligomer formation is highly specific as judged by several criteria, among them the fact that 35S-EmrE can be “pulled out” from a mixture prepared from generally labeled cells. Using this technique, we show that inactive mutant subunits are functionally complemented when mixed with wild type subunits. The hetero-oligomers thus formed display a decreased affinity to substrates. In addition, sulfhydryl reagents inhibit the above hetero-oligomer even though Cys residues are present only in the inactive monomer. It is concluded that, in EmrE, the oligomer is the functional unit.

Transporters are responsible for creating and maintaining the different composition of the cell interior relative to the exterior in both prokaryotic and eukaryotic cells. This is also the case for the solutes’ gradients across internal organelles. Their functioning is therefore highly relevant to maintenance of proper cell homeostasis, and they are targets of action of many drugs. In many cases, they are also responsible for failure of treatment of tumors and infectious diseases because of transporter-mediated multiple drug resistance (1, 2).

The multidrug transporter EmrE, a protein from *Escherichia coli*, provides a unique experimental paradigm for the study of these transporters (3, 4). It is a small multidrug transporter, 110 amino acids long, that extrudes various drugs in exchange with protons, thereby rendering bacteria resistant to these compounds (3, 4). The protein has been characterized, purified, and reconstituted in a functional form (5). Hydrophatic analysis of the EmrE sequence predicts four α-helical transmembrane segments. This model is experimentally supported by Fourier transform infrared spectroscopy studies that confirm the high α-helicity of the protein and by high resolution heteronuclear NMR analysis of the protein structure (6, 7). The transmembrane segments of EmrE are tightly packed in the membrane without any continuous aqueous domain, as was shown by cysteine scanning experiments (8). These results suggest the existence of a hydrophobic pathway through which the substrates are translocated. EmrE has only one membrane-embedded charged residue, Glu-14, which is conserved in more than 50 homologous proteins and was shown to be part of a binding site common to protons and substrates (9). The occupancy of this site by H+ and substrate is mutually exclusive and provides the basis of the simplest coupling for two fluxes (10, 11).

In vitro and in vivo negative dominance studies have been performed to examine the oligomeric state of the protein (12). Co-expression of wild type and non-functional mutants of EmrE resulted in a reduction in the resistance conferred by the transporter. In addition, co-reconstitution of purified non-functional mutants of EmrE with wild type EmrE in proteoliposomes inhibited the wild type transport activity in a dose-dependent manner (12). The results suggested that this inhibition is due to the formation of mixed oligomers in which the presence of nonfunctional subunits cause inactivation. The oligomeric nature of EmrE is further supported by the finding that detergent-solubilized purified EmrE binds between 0.25 and 0.3 mol of the substrate TPP+1 per mol of protein. These data suggest that an oligomeric EmrE complex may form a single TPP+-binding pocket (13). Moreover, EmrE was crystallized in two dimensions, and the projection structure reveals an asymmetric dimer (14).

To further study the oligomeric nature of EmrE, a novel approach was developed. In this method, quantitative monomer swapping is induced in detergent-solubilized EmrE by exposure to 80 °C, a treatment that does not impair transport activity. Oligomer formation is highly specific, as judged by several criteria, among them the fact that the 35S-EmrE can be pulled out from a mixture prepared from generally labeled cells. Inactive mutant subunits are functionally complemented when mixed with wild type subunits. In addition, sulfhydryl reagents inhibit the above hetero-oligomer even though Cys residues are present only in the inactive monomer. It is concluded that, in EmrE, the oligomer is the functional unit.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—*E. coli* JM109 (15) and TA15 strains (16) are used throughout this work. The plasmids used are pT7–7 (17) derivatives for wild type and E14C mutant with (EmrE-His, (13)) or without (5) histidine tag. Plasmid pKK 223–3 (Pharmacia Biotech Inc.) was used for wild type EmrE, the mutants E14C, T18C, and radiolabeled substrate-binding studies suggested that EmrE functions as an oligomer. Projection structure of two-dimensional crystals of the protein revealed an asymmetric dimer. To identify the functional unit of EmrE, a novel approach was developed. In this method, quantitative monomer swapping is induced in detergent-solubilized EmrE by exposure to 80 °C, a treatment that does not impair transport activity. Oligomer formation is highly specific as judged by several criteria, among them the fact that 35S-EmrE can be “pulled out” from a mixture prepared from generally labeled cells. Using this technique, we show that inactive mutant subunits are functionally complemented when mixed with wild type subunits. The hetero-oligomers thus formed display a decreased affinity to substrates. In addition, sulfhydryl reagents inhibit the above hetero-oligomer even though Cys residues are present only in the inactive monomer. It is concluded that, in EmrE, the oligomer is the functional unit.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids**—*E. coli* JM109 (15) and TA15 strains (16) are used throughout this work. The plasmids used are pT7–7 (17) derivatives for wild type and E14C mutant with (EmrE-His, (13)) or without (5) histidine tag. Plasmid pKK 223–3 (Pharmacia Biotech Inc.) was used for wild type EmrE, the mutants E14C, T18C, and 0.3 mol of the substrate TPP. These data suggest that an oligomeric EmrE complex may form a single TPP+-binding pocket (13). Moreover, EmrE was crystallized in two dimensions, and the projection structure reveals an asymmetric dimer (14).
The EmrE Oligomer Is the Functional Unit

Fig. 1. Kinetics of EmrE oligomerization as a function of temperature. In every sample, membranes (13 μg of protein) from cells that over-express EmrE-His and membranes (2.5 μg of protein) from cells with [35S]-EmrE were mixed in Na-buffer containing 0.8% DM, 15, 30, 60, or 240 min at 4°C, 25°C, 60°C, or 80°C. The mixture was cooled to 4°C, and the proteins were immobilized on Ni-NTA beads (1 h, 4°C). The fraction of the [35S]-EmrE immobilized on the Ni-NTA beads is shown.

N11C, Y60F, W63F, and Cys-less (5, 8, 12), and the homologues EM109 and EM121 (9). Plasmid pC-less-XH was used for NhaA-His (18).

Over Expression and Specific Labeling of EmrE—JM109 cells with pKK plasmids and TA15 cells with pGPl-2 (17) and pT7-7 plasmids were grown, and membranes were prepared as in Ref. 5. TA15 cells with the plasmid pGPl-2 (17) and pT7-32 were used for labeling proteins with [35S]methionine as in Ref. 5.

TA15 cells carrying pT7-32 (over-express untagged EmrE) were grown at 30°C in minimal medium supplemented with thiamine (2.5 μg/mL), ampicillin and kanamycin (50 μg/mL), and 0.5% glucose to a cell density of A600 = 0.8. The temperature was then increased to 42°C to induce the TT polymerase; 15 min later, rifampicin (200 μg/mL) was added to a fraction of the cells, and incubation continued for an additional 10 min. Then, the culture was shifted back to 30°C for 40 min. [35S]methionine (1350 Ci/mmol) was added to the cell suspension, and incubation continued for an additional 10 min. Cells were collected by centrifugation, washed with a solution containing 20 mM Tris-Cl, pH 7.5, and 150 mM NaCl, and sonicated three times for 10 s using a probe-type sonicator. Undisrupted cells were removed by centrifugation, and the membranes were then collected by further centrifugation at 213,500 × g for 20 min. The membrane pellet was resuspended in the above buffer, frozen in liquid air, and stored at −70°C.

Formation of Mixed Oligomers—Membranes (7000 dpm/μg protein, 80 μg of protein) from TA15 cells with [35S]-EmrE and membranes (420 μg of protein) from TA15 cells that over-express EmrE-His were mixed and solubilized with at least 15 volumes of Na-buffer (150 mM NaCl, 15 mM Tris-Cl, pH 7.5) containing 0.8% dodecyl maltoside (DM, Anatrace, Inc., Maumee, OH). After incubation at the indicated temperatures for given times, the extract was centrifuged for 1 min at 14,000 rpm to discard precipitates. Washed Ni-NTA beads (Qiagen GmbH, Hilden, Germany; 10 μL of beads/12 μg of membrane protein) were added to the supernatant and incubated at 4°C for 1 h. After pelleting the beads, a sample of the supernatant was taken for measurement of [35S]-labeled radioactivity associated with unbound [35S]-EmrE. To measure the [35S]-EmrE protein immobilized on the beads, they were washed with Na-DM-buffer (Na-buffer containing 0.08% DM) and incubated for 10 min at room temperature with 450 μL of the above buffer containing 150 mM imidazole.

Pull Down of [35S]-EmrE with Tagged EmrE—[3H]Met labeling was done with TA15 cells carrying pT7-32 (over-express EmrE) with (specific labeling) or without (general labeling) rifampicin. Membranes from labeled cells were mixed and solubilized with membranes that over-express EmrE-His, heat treated, and immobilized on Ni-NTA beads as described above. After elution from the beads, the proteins were precipitated with 10% trichloroacetic acid overnight at 4°C. The precipitated proteins were collected by centrifugation, solubilized with sample buffer, separated by SDS-PAGE, and visualized by PhosphorImager FUJIX BAS 100.

Dissociation of Mixed Oligomers—Mixed oligomers of [35S]-EmrE and EmrE-His were formed and immobilized on Ni-NTA beads as described above. To examine the effect of temperature on the dissociation, the beads were resuspended with Na-DM buffer and exposed to the indicated temperature for up to 4 h. The radioactivity associated with the free and bound [35S]-EmrE was measured. To determine the effect of the denaturant detergent SDS, aliquots were resuspended with Na-DM-buffer containing the indicated concentrations of SDS.

[3H]TPP Binding Assays—Proteins immobilized on Ni-NTA beads were assayed for [3H]TPP binding essentially as described (13). Ni-NTA beads were washed twice in distilled H2O and once in Na-DM buffer. The His-tagged proteins were added and incubated at 4°C for 1 h. After washing, the beads were incubated in a total volume of 200 μL for 30 min at 4°C in Na-DM buffer (pH 8) containing 5 or 12.5 mM [3H]TPP (27 or 4 Ci/μmol, respectively, Amersham Bioscience). For negative control reactions, unlabeled TPP (25 μM) was added. The binding reactions were stopped by separating the beads by pulse centrifugation. The bead fraction was then incubated for 10 min at room temperature with 450 μL of Na-DM containing 150 mM imidazole to release the His-tagged proteins and [3H]TPP from the beads. All binding reactions were performed in duplicate.

[14C]Methyl Viologen Uptake into Proteoliposomes with EmrE-His—EmrE-His proteins were purified and reconstituted into proteoliposomes, and uptake of [14C]methyl viologen (11.9 mCi/mmol, Sigma) into the proteoliposomes was measured after 5 min essentially as described (19). Each of the experiments shown in this work was repeated at least twice.

RESULTS

The Rationale—To develop an in vitro assay for studying oligomerization we used untagged [35S]-EmrE that does not spontaneously bind to Ni-NTA beads and unlabeled His-tagged EmrE (EmrE-His). When both are mixed, the degree of oligomer formation is obtained from the amount of radioactivity associated with the beads.

Fig. 1 shows the formation of mixed oligomers of [35S]-EmrE and tagged EmrE as a function of time at various temperatures prior to immobilization. The degree of spontaneous mixing of the detergent-solubilized proteins at low temperature is very low. Treatments, such as high temperatures, that bring about a reversible dissociation of the oligomer prior to immobilization induce also quantitative oligomer mixing. Thus, the maximal amount of mixed oligomers increases with the increase in temperature. At 80°C, more than 95% of the [35S]-EmrE is associated with the beads, and this value is already maximal after about 15–30 min. At 60°C, the final yield is quite similar, but the rate of formation is slower and reaches a maximum after 4 h. At 4 and 25°C, the formation is partial, and only about 10 and 20%, respectively, of the [35S]-EmrE is incorporated even after more than 20 h (not shown). This behavior suggests that, with detergent-solubilized protein, exchange of monomers at the low temperatures is very slow. Some heterogeneity in the...
The EmrE Oligomer Is the Functional Unit

The EmrE oligomer dissociates at the low temperatures (between 8 and 20 °C), more than 50% dissociates after exposure to 60 °C, and practically all the oligomers (about 90%) dissociate after 4 h at 80 °C.

Immobilization of EmrE-His to the beads is done routinely at 4 °C for at least 30 min. The cooling step is necessary for the association of the oligomers since very little mixing was observed when the immobilization was performed at 80 °C (Fig. 2B). As is seen from the results, the association process itself is faster than the minimal 30 min required for quantitative immobilization to the beads.

Other denaturing agents, such as the detergent SDS, also induce dissociation as a function of the concentration (Fig. 2C). Already at 0.05% SDS, full dissociation is observed after 20 min. On the other hand, detergents, such as DM, do not induce dissociation even at concentrations as high as 1% (not shown).

We also tested other agents that are directly interacting with EmrE, such as protons and substrates. pH conditions at the range between 6.2 and 9 and presence of the substrates ethidium bromide, methyl viologen, and TPP+ (at 200, 200, and 20 μM, respectively) do not induce dissociation of the oligomers (not shown). These results suggest that the oligomeric structure of EmrE is very stable at a wide range of conditions.

EmrE Is Functional after Exposure to High Temperature—Detergent-solubilized EmrE binds the substrate TPP+ with high affinity (13), and upon reconstitution, it accumulates methyl viologen in a ΔpH-driven process (5). Exposure to 80 °C for 15 min caused only a 20–30% decrease in the ability of EmrE to catalyze either function (Fig. 3). It is concluded that EmrE is resistant to this extreme temperature and does not irreversibly denature under these conditions. Therefore, this treatment can be used to induce generation of mixed oligomers and to study their functional properties.

The Oligomer Formation Is Highly Specific—The experiments described in Fig. 4A show that the association of 35S-EmrE to the beads is fully dependent on the presence of tagged EmrE. While mixing with membranes prepared from cells expressing tagged EmrE resulted in association of almost 80% of the labeled protein with the beads (lane I), addition of membranes prepared from control cells without tagged EmrE (lane IV), or no membranes (lane V) caused only a small association with the beads (only 3% of the labeled protein). In addition, another unrelated His-tagged protein such as the E. coli Na+/H+ antipporter (NhaA-His), induces practically no association (lane III). To show that no other protein components in the membrane are involved or required for the mixing of the oligomers, purified tagged EmrE was used (lane II). Also in this case a quantitative mixing was observed, and about 60% of the labeled EmrE was associated with the beads.

To further stress the exquisite specificity of the oligomer formation, membranes were prepared from cells in which overexpression of untagged EmrE was induced but were generally labeled with [35S]methionine (Fig. 4B, lane I) as opposed to the same membranes in which the labeling was specific (Fig. 4B, lane IV). Membranes of both kinds were solubilized, mixed with tagged EmrE, and heat treated as described above. After immobilization on the Ni-beads, the proteins were eluted, separated on SDS-PAGE, and analyzed for radioactivity. As seen in lanes II and V, the results are practically indistinguishable, and the bulk of the labeled protein that bound to the Ni-beads, in both cases, is EmrE. The treatment of the generally labeled membranes results in a specific pull down of EmrE and, actually, provides a new way for purifying small amounts of untagged protein. Again, in both cases, binding to the beads is completely dependent on the presence of His-tagged EmrE (lanes III and VI). The small amounts of labeled protein other than EmrE that bound to Ni-beads (lane II) are not dependent
The EmrE Oligomer Is the Functional Unit

FIG. 3. Exposure to high temperature has little effect on EmrE activity. A, membranes (60 μg of protein) from cells that over-express EmrE-His were solubilized in Na-buffer containing 0.8% DM for 15 min at 20°C or 80°C. Next, the tagged proteins were purified and reconstituted into proteoliposomes, and uptake of [14C]methyl viologen (11.9 μCi/mmol) into the proteoliposomes was measured for 5 min essentially as described (5). B, membranes (1 μg of protein) from cells that over-express EmrE-His were solubilized in Na-buffer containing 0.5% DM for 15 min at 20°C or 80°C. The tagged proteins were immobilized on Ni-NTA beads and [3H]TPP immobilized on Ni-NTA beads and [3H]TPP binding was measured as described under “Experimental Procedures.”

on the presence of tagged EmrE (compare with lane III) and probably reflect nonspecific interaction with the beads. It is concluded that the oligomer formation is highly specific: tagged EmrE forms oligomers only with EmrE (35S-labeled-untagged) and can recognize it in a mixture of membrane proteins.

Another way to demonstrate the specificity of the formation of the oligomer in this in vitro method is to test whether other proteins will compete with the untagged 35S-EmrE (present only at tracer amounts). As expected, untagged-untagged EmrE added at increasing amounts to the mixture prior to the heat treatment, decreases the amount of 35S-label associated with EmrE-His (Fig. 5). The competition was also performed with purified EmrE (Fig. 5, inset). Fifty percent inhibition is detected at about 100 nM EmrE suggesting an approximate apparent affinity for the oligomer in this order of magnitude.

This assay was also used to test whether a series of inactive mutants are capable of forming oligomers. We competed between untagged EmrE mutants, and 35S-EmrE, at the stage when the mixed oligomers are formed. Addition of increasing amounts of membranes prepared from cells expressing various EmrE mutants (Fig. 5) brought about a decrease in the association of 35S-EmrE to the Ni-beads. The decrease was very similar for wild type and the non-functional mutants: E14C, I11C, T18C, Y60F, and W63F (Fig. 5, the results with only E14C and Y60F are shown for the sake of clarity). The slight differences in inhibition can be accounted for by the different expression levels of the mutants. Therefore, it is concluded that the mutants tested form oligomers with the tagged EmrE with affinities in the same order of magnitude as that of wild type EmrE. These results suggest that the lack of function in the mutations tested is not due to impairment in their ability to form oligomers.

Two other Smr proteins from E. coli, EM109 and EM121 (9), do not form mixed oligomers with EmrE. Thus, when membranes of cells that over-express these two proteins were added as above, to 35S-EmrE and EmrE-His, there was no effect on the level of association of 35S-EmrE to the Ni-beads (Fig. 5, shows only the results of EM121; the results with EM109 are similar). These results stress again the specificity of oligomer formation as measured in this assay since these homologous proteins (32 and 33% identity, respectively) do not form mixed oligomers. Other homologues with higher sequence identity (41–58%) are capable of forming hetero-oligomers (9).

Functional Implications of Oligomer Formation: Functional Complementation—The in vitro method was also used to examine whether each one of the EmrE subunits functions independently or in a concerted form. Negative dominance studies have suggested that the oligomer is the functional unit (12). In these studies, oligomers formed by mixing organic solvent extracts of wild type and inactive mutants were shown to be inactive when tested for pH-driven accumulation of [14C]methyl viologen in proteoliposomes (12). The monomer mixing technique described in the present work and the ability to assay high affinity binding in the detergent-solubilized protein allow us an in-depth analysis of the negative dominance phenomenon. We generated mixed oligomers of EmrE-His and the non-functional untagged mutant E14C, and we showed a dose-dependent inhibition of [3H]TPP binding (Fig. 6A). As expected from the previously published experiments, when an excess of E14C membranes were added inhibition was detected, which further increased with increasing amounts of E14C. The nature of this inhibition was additionally studied by analyzing the kinetic parameters of the residual [3H]TPP binding at the above ratio of mutant to wild type protein. While EmrE-His binds TPP with an affinity of 2.8 ± 1.0 nM (Bmax = 0.9 pmol TPP/μg EmrE-His membrane protein) the mixed oligomer shows a biphasic behavior: Kd1 = 1.9 nM and Kd2 = 40.7 nM, Bmax = 0.08 and 0.39 pmol TPP/μg EmrE-His membrane protein, respectively (Fig. 6B).

These results demonstrate that the mixing brings about a decrease in the total amount of binding sites (from 0.9 to 0.47) and that most of the remaining binding sites display a lower affinity to TPP. Therefore, in this experiment at least three discrete populations are distinguished based on their ability to bind TPP: one similar to wild type, one with a 20-fold lower affinity, and a nonfunctional one. These findings suggest that hetero-oligomers with a certain ratio of E14C:WT bind TPP with different kinetic properties. To further characterize this population and to try to detect hetero-oligomers with potentially different affinities, we designed a more sensitive protocol. Thus, we performed the reverse experiment in which the tagged protein is the inactive E14C mutant, while the untagged is the wild type. As previously shown, E14C does not bind TPP (Fig. 7). Mixing with increasing amounts of the untagged wild type EmrE results in a dose-dependent increase of the TPP binding that levels off at a 4-fold excess (Fig. 7). In these experiments we detected only a single affinity of TPP to the hetero-oligomers that was 36 nM, practically identical to that observed in the inverse experiments in which EmrE-His binding was inhibited with saturating amounts of E14C. The affinity was identical whether determined at a 1:1 or a 10:1 ratio WT:E14C. These findings demonstrate that the amount of the species that binds with low affinity increases with increasing amounts of wild type membranes. It is suggested that this...
is the population with a single Cys at position 14. We were not able to identify multiple affinities, suggesting that hetero-oligomers with more than one Cys residue at position 14 bind with very low affinities or are non functional.

The experiments described above support the notion that the functional properties of the oligomer are a result of the interaction between individual monomers. An elegant proof of this contention is provided in experiments in which a functional hetero-oligomer is generated so that it contains Cys residues only in the inactive monomer. This is achieved when tagged E14C was mixed with untagged Cys-less EmrE. When this species is challenged with the sulphydryl reagent N-ethylmaleimide (NEM), a dose-dependent inhibition is observed (Fig. 8). A 60% inhibition is observed at 100 μM NEM that increases to about 75% at 300 μM NEM and levels off thereafter. The remaining binding activity (about 25%) shows a decreased affinity to TPP+ of about 90 nM. Neither the wild type nor Cys-less EmrE are significantly inhibited by NEM even at 1 mM. We conclude that NEM reacts with Cys at position 14 in the inactive mutant and affects the affinity of the oligomer.

DISCUSSION

The oligomeric state of ion-coupled transporters has been investigated in a number of cases. The approaches used are based on analysis of the particle size of the protein in the membrane (20, 21) or on detergent-solubilized preparations (see for example Refs. 22 and 23). Evidence for oligomer formation has been presented in many instances, and the functional relevance of the oligomerization has been documented in a few cases (18, 22–24). The results presented here describe a novel in vitro system for the study of oligomerization and its functional implications. EmrE oligomers are stable in the detergent-solubilized preparation and dissociate only after exposure to high temperature or strong denaturing reagents such as the detergent SDS. An experimental protocol for generating mixed oligomers was designed based on an initial dissociation step induced by exposure to high temperature for short periods and rapid association of the monomers in solution at a lower temperature. The exposure to high temperature had little effect on either substrate binding or ΔpH-driven uptake in proteoliposomes. EmrE provides, therefore, a unique experimental paradigm not only because of its size but also because of its stability to denaturing agents such as temperature, organic solvents (5), and SDS-urea (3).

The results demonstrate that EmrE forms homo-oligomers since 35S-EmrE binds to Ni-NTA beads only through formation of oligomers with EmrE-His. No other proteins seem to be required for this process because oligomerization is detected also when purified EmrE-His is used. In addition, competition experiments were performed with unlabeled untagged wild...
Procedures

[3H]TPP

80°C mixed and solubilized in Na-buffer containing 0.8% DM for 15 min at 80°C. Cells and increasing amounts of membranes from E14C EmrE cells were

to wild type, one with a 20-fold lower affinity, and a nonfunctional type. These findings suggest that hetero-oligomers with one Cys residue at position 14 bind TPP+

carboxyl moieties at position 14 are in close proximity and form a charge cluster (10, 11), the negative charge being essential for substrate binding. The results described above suggest that type EmrE showing an apparent affinity of interaction in the 100–200 nm range. Oligomer formation appears to be exquisitely specific as no formation is detected with unrelated tagged membrane proteins such as NhaA-His and even with other Smr proteins from E. coli that show a distinct homology to EmrE. The lack of interaction of EmrE with other Smr proteins from E. coli supports the contention that EmrE is functional in vivo only as a homo-oligomer. In the case of Smr proteins from other organisms, it has been suggested that they can function as hetero-oligomers based on a synergistic effect on resistance phenotype when two proteins are co-expressed (25, 26).

We have recently shown that the basic oligomeric structure detected in two-dimensional crystals of EmrE is a dimer (14), but it is possible that the functional unit of the protein is a higher degree oligomer formed by two or more dimers. Previous evidence from negative dominance experiments and from ligand binding measurement to purified EmrE were consistent with a trimeric structure but did not rule out the possibility that EmrE functions as a dimer or a tetramer (12, 13). The experiments described here demonstrate that in the mixed oligomers the inhibitory effect of E14C is on the substrate binding site. The kinetic analysis of this effect revealed three distinct species based on their binding properties: one similar to wild type, one with a 20-fold lower affinity, and a nonfunctional one. These findings suggest that hetero-oligomers with one Cys residue at position 14 bind TPP+ with different kinetic properties. To further characterize this population and to try to detect hetero-oligomers with potentially different affinities, we designed a more sensitive protocol. In these experiments, the tagged protein is the inactive E14C mutant, while the untagged is the wild type. The functional complementation detected reveals only one functional species with a low affinity identical to the one measured in the negative dominance experiments. In our most recent model, it was suggested that the carboxyl moieties at position 14 are in close proximity and form a charge cluster (10, 11), the negative charge being essential for substrate binding. The results described above suggest that replacing one of the charges in this site has a marked effect on the affinity toward the substrate, while replacing more than one yields a non-functional protein.

That the binding site is contributed by each of the subunits was demonstrated also by experiments in which a hetero-oligomer with Cys residues only in the inactive monomer was challenged with NEM, a sulfhydryl reagent. NEM inhibited the activity of this hetero-oligomer even though it does not have any effect on the activity of the wild type. Therefore, the inhibitory effect described must be due to an interaction with the
Cys residue at position 14 in the inactive subunit. NEM inhibits the activity by lowering even further the affinity (90 nM), and it may be doing so by modifying the environment around the cluster. This novel finding allows insertion of various compounds to further probe the binding site.

The novel method described here for generation of mixed oligomers has provided significant information on the functional size of the protein. It also allows generation of hetero-oligomers with desired compositions, and it could be of general use with other proteins as well. In addition, as was shown here, we have developed an unexpected means to gain access to the vicinity of the binding site.

Acknowledgment—We thank Michal Sharoni for performing some of the experiments.

REFERENCES

1. Nikaido, H. (1994) Science 264, 382–388
2. Gottesman, M. M., and Pastan, I. (1993) Annu. Rev. Biochem. 62, 385–427
3. Schuldiner, S., Granot, D., Mordoch, S. S., Ninio, S., Rotem, D., Soskin, M., Tate, C. G., and Yerushalmi, H. (2001) Biochimie 83, 3165–3169
4. Schuldiner, S., Granot, D., Steiner, S., Ninio, S., Rotem, D., Soskin, M., and Yerushalmi, H. (2001) J. Mol. Microbiol. Biotechnol. 3, 133–137
5. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1999) J. Biol. Chem. 274, 610–619
6. Steiner, Mordoch, S., Granot, D., Lebendiker, M., and Schuldiner, S. (2000) J. Biol. Chem. 274, 19480–19486
7. Ninio, S., Rotem, D., and Schuldiner, S. (2001) J. Biol. Chem. 274, 6856–6863
8. Arkin, I., Russ, W., Lebendiker, M., and Schuldiner, S. (1996) Biochemistry 35, 7233–7238
9. Schweiger, M., Lebendiker, M., Yerushalmi, H., Coles, M., Groger, A., Schwarz, C., Schuldiner, S., and Kessler, H. (1996) Eur. J. Biochem. 254, 511–518
10. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (1999) J. Biol. Chem. 274, 6856–6863
11. Yerushalmi, H., Lebendiker, M., and Schuldiner, S. (2001) J. Biol. Chem. 276, 12744–12748
12. Friesen, R. H., Knol, J., and Poolman, B. (2000) J. Biol. Chem. 275, 33527–33533
13. Kilic, F., and Rudnick, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 3110–3115
14. Veenhoff, L. M., Heuberger, E. H., and Poolman, B. (2001) EMBO J. 20, 2345–2346
15. Lee, A., Mao, W., Warren, M. S., Mistry, A., Hoshino, K., Okumura, R., Ishida, H., and Lomovskaya, O. (2000) J. Bacteriol. 182, 3142–3150
16. Jack, D. L., Storms, M. L., Tchieu, J. H., Paulsen, I. T., and Saier, M. H., Jr. (2000) J. Bacteriol. 182, 2311–2313