A Mechanism to Alter Reversibly the Oligomeric State of a Membrane-bound Protein Demonstrated with Escherichia coli EII<sup>mtl</sup> in Solution<sup>*</sup>

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This paper reports that the aggregation state of a membrane protein can be changed reversibly without the use of chaotropic agents or denaturants by altering the attractive interactions between micelles of polyethylene glycol-based detergents. This has been documented using mannitol permease of Escherichia coli (EIImtl), a protein whose activity is dependent on the dimerization of its membrane-embedded domains. We show that the driving force for the hydrophobic interactions responsible for the dimerization can be increased by bringing the protein into a less polar environment. This can be done simply and reversibly by increasing the micelle cluster size of the solubilizing detergent since the micropolarity in the micelle decreases upon clustering and is directly related to the cluster size.

The micelle cluster size was varied at a fixed temperature by adding sodium phosphate or a second detergent with a distinct clustering behavior, and the changes were quantified by quasi-elastic light scattering and by determining the cloud point or demixing temperature (T<sub>d</sub>) of the detergent. Maximal EIImtl activity was found when no micelle clustering occurred, but the activity gradually decreased down to 5% of the maximal activity with increasing cluster size. The inactivation was found to be completely reversible. The kinetics of heterodimer formation were also significantly affected by changes in the micelle cluster size as expected. Increasing the cluster size resulted in faster formation of functional heterodimers by increasing the rate of homodimer dissociation. This phenomenon should be generally applicable to controlling the oligomeric state of membrane-bound proteins or even water-soluble proteins if their subunit association is dominated by hydrophobic forces.

It is well documented that the micropolarity of PEG-based detergents decreases when they form clusters and that this decrease is directly related to the micelle cluster size (1–3). In principle, this decrease in micropolarity should be able to be used to transfer a membrane protein, solubilized by such a detergent, to a less polar environment. Only PEG-based detergents show this clustering behavior. It can be induced by heating where, at a certain temperature, the cloud point or demixing temperature (T<sub>d</sub>), the cluster size reaches a value which initiates separation of the micellar phase into two new micellar phases, one consisting of the micellar clusters and one containing a low concentration of micelles (2). Phase separation or demixing of PEG-based detergents into two new micellar phases at T<sub>d</sub> upon heating has been studied in detail with various techniques such as light, neutron and x-ray scattering, viscosity measurements, and NMR spin lattice relaxation (2, 4–8). Such studies suggest that the intermicellar forces become stronger upon heating due to a decrease of the hydration of the PEG chains. This leads to stronger van der Waals interactions between the micelles and the formation of micelle clusters. It is believed that the size of the micelle does not change upon heating (7), but the size of the clusters increases asymptotically and follows a power-law given by (T<sub>d</sub> − T)/T<sub>d</sub> (4, 6). The attractive forces between micelles can also be increased at a fixed temperature, T, by the addition of certain inorganic salts, especially phosphate or fluoride salts, or by mixing with a detergent with a lower T<sub>d</sub> (2). Whether one increases T or lowers T<sub>d</sub>, the result in the same, the interval T<sub>d</sub> − T decreases, and as the two approach one another, the micelle cluster size increases until, at T = T<sub>d</sub>, phase separation occurs. This demixing property has been used in biochemical studies to separate hydrophobic proteins from more hydrophilic ones (9). Hydrophobic proteins concentrate in the detergent-rich phase upon heating the detergent solution above T<sub>d</sub>. After centrifugation, this phase can be easily separated from the aqueous phase, containing the hydrophilic proteins. Triton X-114 with T<sub>d</sub> = 22 °C is often used in such procedures.

Here we demonstrate that these same intermicellar attractive forces can be used in a more subtle way to disrupt the hydrophobic forces responsible for subunit interactions and thereby reversibly control the association state of EIImtl in PEG-based detergents. EIImtl is inactive as a monomer and phosphorylates mannitol when in the dimeric state. Hydrophobic forces are involved in the dimerization process (10). Both the activity of EIImtl and the rate of formation of EIImtl heterodimers can be controlled by choosing a specific micellar cluster size. The results are explained by relating the decrease...
in micropolarity of the micelles upon clustering with the decrease in the driving force for hydrophobic bonding, the interactions responsible for EIImul dimerization.

**EXPERIMENTAL PROCEDURES**

**Materials**—dPEG, C10E5, C10E6, and C10E4 were supplied by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). dMal was from Sigma. Na3PO4 solutions were adjusted to pH 7.6 with concentrated H3PO4.

Q-Sepharose Fast Flow and S-Sepharose Fast Flow were from Pharmacia (Sweden); hexyl-agarose was from Sigma. D-[1-3H]Mannitol (976.8 GBq/mmol) was from NEN Life Science Products. EI and HPr were purified as described previously (11–13). All other reagents were analytical grade. The purification of EIImul was as described previously for EIImul (C884S) (14).

**Mannitol Phosphorylation Assays**—The PEP-dependent mannitol phosphorylation activity of EIImul was measured as described (15). The assay buffer contained 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgCl2, 5 mM PEP, 0.25% (v/v) detergent, and eventually Na3PO4 or C10E5 to lower the Tc. The Tc measurements were performed on the same solutions. The PEP-dependent phosphorylation activity of EIImul was determined by incubating the enzyme for 5–10 min at 30 °C in 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgCl2, 5 mM PEP, 0.25% (v/v) detergent, 20 μM HPr, 0.33 μM EI, and eventually Na3PO4 or C10E5 to lower the Tc. The reaction was started after the incubation period by adding [3H]mannitol with a final concentration of 60 μM (final volume 100 μl). At four different times, 20 μl aliquots were taken, and the amount of mannitol-1-phosphate was quantified.

**Concentration Determinations on EIImul Samples**—The EIImul concentrations were determined by flow dialysis which quantitates the number of mannitol binding sites (16), assuming one high affinity binding site (Ka = 100 nm) per EIImul dimer in accordance with the observations of Pas et al. (14).

**Light Scattering Experiments**—Light scattering experiments were performed at 30 °C by using a DynaPro-801TC instrument (Protein Solutions Inc., Charlottesville, VA), equipped with a thermostated cell. Detergent (0.25%, v/v) was dissolved in 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgCl2, and 5 mM PEP. In one case, 0.5% dPEG was used since no stable signal at 0.25% was observed. Solutions were filtered through 0.1 μm Anotop10 filters (Whatman). Data were analyzed using the software supplied by the manufacturer. Data could be resolved by the theoretical single exponential autocorrelation function (monomodal analysis), indicating that the solutions were monodisperse. Each sample was measured at least seven times. Standard deviations in Rh were 0.1 nm or less.

Cloud points (± 0.5 °C) were determined by heating a detergent solution in a test tube with a thermometer.

**RESULTS**

**Effect of Micelle Cluster Size on the Activity of EIImul**—Micelles clustering is reflected in an increase in the apparent hydrodynamic radius (Rg) of the micelle, and this occurs as the temperature of the solution approaches the cloud point (Tc). The phosphorylation activity of EIImul has been determined at 30 °C in PEG-based detergent mixtures with various cloud points, and the apparent hydrodynamic radii of the micelles clusters of these solutions under the assay conditions has been determined by quasi-elastic light scattering experiments. The Tc of PEG-based detergents was lowered by increasing the concentration of sodium phosphate. Addition of up to 250 mM Na3PO4 (pH = 7.6) lowered the Tc of dPEG from 58 to 33 °C with a corresponding increase in Rh from 3.4 to 8.2 nm (Fig. 1A, C). A plot of the enzyme activity against Rh shows that increased values of Rh result in a lowering of the enzyme activity down to 10% of the activity found in dPEG with Rh = 3.4 nm.
The decreasing EIImtl activity with increasing $T_d$ results in dissociation of active dimers into less active or inactive monomers. Increasing the EIImtl concentration shifts, by mass action, the equilibrium back to the active, dimeric form.

The reversibility of the change in association state upon a change in $T_d$ has been demonstrated in C10E5 buffer. EIImtl (0.8 mM) was incubated for 10 min at 30 °C in buffer containing 120 mM Na3PO4 ($T_d = 34$ °C) to cause the protein to dissociate. One portion of this solution was then diluted 10-fold with assay buffer containing the same Na3PO4 concentration while another portion was diluted with assay buffer lacking Na3PO4. After 10 min at 30 °C, the phosphorylation reaction was started by adding 60 μM mannitol. As expected, a low activity (240 nM/min/mM enzyme) was observed for the enzyme incubated with buffer containing 120 mM Na3PO4 ($T_d = 34$ °C) and Na3PO4 resulting in final Na3PO4 concentrations of 0 (○), 215 (□), 235 (△), and 250 mM (●). The $R_b$ values were 3.4, 6.4, 7.3, and 8.2 nm, respectively, and the corresponding $T_d$ were 58, 37, 35, and 33 °C. Final mannitol concentration was 60 μM.

Fig. 3 presents the effect of a rapid change in $T_d$ on the EIImtl activity. The enzyme was preincubated in a dPEG buffer with high $T_d$ (58 °C) along with PEP, E1, and HPr to generate phosphorylated EIImtl (P-EIImtl) and then diluted into buffers with mannitol and dPEG with the same or lower $T_d$ results. Therefore, the enzyme concentration shifts, by mass action, the equilibrium back to the active, dimeric form.

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The results presented so far show a reversible dissociation of EIImtl dimers into less active monomers upon time. The reaction was started with mannitol, and the accumulation of mannitol-1-phosphate over time was monitored. In detergent solutions with a high Td (58 °C), a slow initial activity was observed that gradually increased in time and then became constant (Fig. 4, ●). When the Td was decreased, initial activities increased, resulting in faster attainment of a constant PEP-dependent phosphorylation activity (○). In a detergent solution with a Td of 35 °C, a high and constant activity was observed immediately (Fig. 4, ×). These results suggest a more rapid formation of functional heterodimers from inactive homodimers in detergent solutions with a lower Td.

The following experiments were performed to examine the formation of heterodimers in more detail (Fig. 5). EIImtl (G196D) and IICmtl were incubated for 10 min in buffer containing 25 mM Na3PO4, 5 mM DTT, 5 mM MgCl2, 5 mM PEP, 0.25% (v/v) dPEG, 20 μM HPr, 0.33 μM EI, and 0 μM (●), 90 μM (○), or 235 mM Na3PO4 (●) with Td = 58, 50, and 35 °C corresponding to Rd = 3.4, 4.1, and 7.3 nm, respectively. The reaction was started with 60 μM mannitol. Inset, schematic representation of the complementation of IICmtl (1) and EIImtl(G196D) (2) yielding EIImtl (G196D)/IICmtl heterodimers (3). The nomenclature of the EIImtl domains (A, B, C) has been used.

The samples were diluted into dPEG buffers with a lower Td, the time dependence showed two phases. (i) In the first 4–5 min, the specific activity gradually decreases. (ii) After 5 min, the specific activity was stable, the lower the Td then the lower the activity.

Since the new Td values were reached immediately after mixing (see "Experimental Procedures"), these phases most likely reflect the time-dependent changes in the association state of the enzyme. During the first phase, the concentration of monomer in solutions with lower Td increases until a new monomer-dimer equilibrium is established itself. After 5 min, the new equilibrium is established, and the rates are linear with time. The results presented so far show a reversible dissociation of active EIImtl dimers into less active monomers upon increasing Rd, or correspondingly decreasing Td. In the next section, the effect of variation of Td on the formation of heterodimers will be presented.

**Effect of Variation in Micelle Cluster size in Complementation Assays—**The influence of micelle cluster size on the kinetics of heterodimer formation of EIImtl has been studied by using two EIImtl mutants, each of which lack PEP-dependent activity but become active upon formation of a heterodimer (complementation assay, see Fig. 4, inset). One such couple is EIImtl (G196D), with drastically reduced binding affinity for mannitol, and IICmtl, which is inactive by virtue of the missing A and B domains (18). Upon formation of a heterodimer, the phosphoryl group can proceed from PEP via HPr and EI and the A and B domains of EIImtl to mannitol bound in the C domain (see “Discussion”). Fig. 4 follows the rate of appearance of phosphorylation activity when these two enzymes are incubated in detergent of varying Td. EIImtl (G196D) and IICmtl were incubated for 10 min in phosphorylation buffer with dPEG and various concentrations of Na3PO4 shifting the Td between 58 and 35 °C (Rd = 3.4–7.3 nm). The reaction was started with mannitol, and when the enzyme is solubilized in 0.25% dPEG (Td = 58 °C) or C18E6 (Td = 35 °C), mannitol were omitted during the incubation of the two enzymes, and the reaction was started with these two components, a similar pattern was found as when the reaction was started with mannitol (Fig. 5B, ×). The similarity in activity profiles of ● and × shows that prior phosphorylation of EIImtl does not affect the process of heterodimer formation as probed under the conditions used.

Finally, we have investigated the formation of heterodimers in n-decy-β-D-maltopyranoside (dMal), a detergent which does not form micelle clusters and experience phase separation. The specific activity of EIImtl in 3 mM dMal is the same as when the enzyme is solubilized in 0.25% dPEG (Td = 58 °C) or C18E6 (Td = 35 °C), which suggests that the specific activity of the homodimers is not altered by the presence of dMal. In the presence of dPEG, the specific activity of EIImtl increased with time, indicating that the formation of heterodimers was faster in the absence of dPEG. In detergent solutions with a low Td, the specific activity of the heterodimers was found (Fig. 5A, ●). But if the initial solution was diluted in buffer with high Na3PO4 to produce a low Td (37 °C), maximal phosphorylation activity was observed immediately (Fig. 5A, ×). Similarly, if the incubation was performed under low Td conditions and the assay at either high or low Td conditions, maximal initial activity was found immediately (Fig. 5A, ○ and ■, respectively). Therefore, as long as both enzymes have been incubated with each other under low Td conditions, formation of heterodimers is complete within the 5-min incubation period and does not reverse when the Td is subsequently increased. Although more inactive monomers are expected in detergent solutions with a low Td (Figs. 1 and 3), the high protein concentration used in these experiments will result in high heterodimer concentrations. These experiments show that formation of heterodimers is very fast when the Td of the buffer is low; moreover, the specific activity of the heterodimer is not affected at high enzyme concentrations when the Td is varied between 37 and 63 °C (Rd = 6.4–3.4). Incubation of EIImtl with mannitol has been reported to stimulate the formation of heterodimers (19) and could also be expected to stimulate heterodimer formation. To test this, EIImtl (G196D) and IICmtl were incubated for 10 min at 30 °C in assay buffer with dPEG (Td = 58 °C) that included PEP, EI, and HPr. Upon starting the reaction with mannitol, a low initial activity was found (Fig. 5A, ○). However, when mannitol replaced PEP in the incubation step and the reaction was started with PEP, maximal activity was found almost immediately, indicating that formation of heterodimer was nearly completed at the moment that PEP was added (Fig. 5B, ○). When both PEP and mannitol were omitted during the incubation of the two enzymes, and the reaction was started with these two components, a similar pattern was found as when the reaction was started with mannitol (Fig. 5B, ×).
FIG. 5. Dependence of the rate of appearance of the phosphorylation activity of EII
(G196D)/IIC mtl heterodimers on the apparent hydrodynamic radii \( R_g \) of the
micelles and the presence of mannitol and/or PEP. A, EII mtl (G196D) (200 nM) and
IIC mtl (1.45 μM) were incubated at 30 °C for 10 min in buffer containing 0.25% dPEG, 5 mM DTT,
5 mM MgCl₂, 5 mM PEP, 20 μM HPr, 0.33 μM EI, and after 5 min at 30 °C, the reaction was
started with 60 μM mannitol (final \( R_g \) and \( T_w \) are 3.4 nm and 58 °C, respectively). C, solution 1 was
diluted 12-fold into 25 mM Na₃PO₄, pH 7.6, 5 mM DTT, 5 mM MgCl₂, 5 mM PEP, 0.25% dPEG, 20 μM HPr,
0.33 μM EI, and after 5 min, the reaction was started with mannitol (final \( R_g \) and \( T_w \) are
6.4 nm and 37 °C, respectively). ○, solution 2 was diluted 12-fold into 25 mM Na₃PO₄, pH 7.6, 5 mM DTT,
5 mM MgCl₂, 5 mM PEP, 0.25% dPEG, 20 μM HPr, 0.33 μM EI, and after 5 min at 30 °C, the
reaction was started with 60 μM mannitol (final \( R_g \) and \( T_w \) are 3.4 nm and 58 °C, respectively).
B, dilution effects of detergents on the PEP-dependent phosphorylation assay. The reaction was started with
60 μM mannitol (●), 5 mM PEP (○), or 60 μM mannitol and 5 mM PEP (×).

Detergents used in the study were Lubrol PX and dPEG. EIImtl has been
used to study the domain structure and oligomerization state of EIImtl. While some work was carried out on
proteoliposomes of EIImtl, most of these studies have been performed with a PEG-based
detergent, especially Lubrol PX and dPEG. EIImtl has been observed both as a monomer and a dimer (19, 21, 26).
The crucial contacts resulting in the dimer appear to be between the hydrophobic C domains (23, 27). Stephen and Jacobson (19)
found that, upon mild extraction of the enzyme from vesicles, the percentage of dimer increased with increasing ionic
strength but decreased upon introduction of the PEG-based
detergent, Lubrol PX, or mannitol or upon phosphorylation of
the enzyme. Kinetic experiments have shown that the dimer is
primarily responsible for the PEP-dependent phosphorylation
and mtl/mtl-P exchange (10, 28). The formation of het-

erodimers is another approach used to show that EIImtl dimers
are functional (18, 27, 29, 30). Mutants of EIImtl, each inactive
by virtue of a mutation in the A, B, or C domain, could be

reactivated by mixing with another mutant form carrying the
mutation on another domain (see Fig. 4, inset). Apparently,
the phosphoryl group can cross the dimer interface when proceed-
Effect of Cloud Point of Detergents on EIImtl Activity

The following evidence indicates that an increased tendency of the micelles to form clusters (high \( R_b \) conditions) induces monomerization of EIImtl dimers. 1) A lowering of the EIImtl activity was observed with increasing \( R_b \). 2) The specific activity of EIImtl, under conditions of high \( R_b \), increased with increasing enzyme concentration. A higher percentage of (active) dimers is expected upon an increase in enzyme concentration, due to mass action. Under conditions of minimal \( R_b \) (maximal \( T_d \)), maximum activity was observed at all EIImtl concentrations monitored, indicative of a completely dimeric enzyme. The increase of specific activity which Boer et al. (27) observed upon addition of high concentrations of IICmtl to active EIImtl can also be explained in these terms; a population of EIImtl monomers were present under their specific detergent conditions, which were titrated by high concentrations of IICmtl to form active heterodimers. 3) The rates of heterodimer formation, from two homodimers, were significantly increased under conditions of high \( R_b \) due to the increased rate of dissociation of the homodimers. In line with this, the kinetics of heterodimer formation were also increased by the introduction of mannitol, a substrate known to dissociate EIImtl (19).

The relationship between the EIImtl activity and the \( T_d \) of dPEG or C10E5 was found to be similar, the lowering of \( T_d \) was caused by C10E4 or Na3PO4 (Fig. 1). This, and the observation that the EIImtl activity at low \( T_d \) can be completely converted to the high activity found in buffer with a high \( T_d \), supports the view that the enzyme is sensitive to changes in the micellar properties rather than the chemical composition of the detergent. EIImtl exhibits maximal activity when solubilized in dPEG, C10E5, or C10E6 (not shown). The micellar size of dPEG (\( T_d = 58^\circ \text{C} \)), C10E5 (\( T_d = 44^\circ \text{C} \)), and C10E6 (\( T_d = 62^\circ \text{C} \)) at 30 °C, where almost no clustering of micelles is expected, differs significantly (\( R_b \) is 3.4 nm, 4.9 nm, and 2.9 nm, respectively). Apparently, the size of the micelle is not important for the monomer to dimer equilibrium. The concentration of micelles is 4 orders of magnitude higher than the enzyme concentration used in these experiments. Clustering of micelles, including the ones containing EIImtl, will result in partial solvation of the EIImtl-detergent micelles by other detergent micelles instead of by bulk water. This shift in solvation is expected to increase with increasing micelle cluster size. Since hydrophobic forces are involved in the formation of the EIImtl dimer (10, 19), the shift to a more hydrophobic environment most likely explains the dissociation of dimers.

Recently, Boer et al. (18) demonstrated that the simultaneous expression of EIImtl (G196D) and IICmtl in E. coli resulted in cells that were able to take up mannitol. No mannitol uptake was observed if the mutants were expressed separately. This experiment showed that heterodimer formation between EIImtl (G196D) and IICmtl occurs in vivo. Heterodimer formation also proceeds in dPEG and C10E5 and is facilitated by an increase of \( R_b \) (decrease in \( T_d \)). Almost no heterodimer formation was found in dMal although the wild-type enzyme is highly active when solubilized in this detergent. Therefore, PEG-based detergents are probably the best class of detergents for mechanistic investigations on EIImtl since the activity is comparable with the activity for EIImtl in vesicles (31), and heterodimer formation is readily achieved in these detergents.

In conclusion, a “new” detergent parameter relevant for membrane protein chemistry, i.e., the tendency of micelles to cluster, has been shown to control the oligomerization state of EIImtl. Proper control of this variable can result in almost complete monomerization or dimerization of the protein. It provides a unique tool to study mechanistic aspects of membrane protein oligomerization.

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