The poor stability of membrane proteins in detergent solution is one of the main technical barriers to their structural and functional characterization. Here we describe a solution to this problem for diacylglycerol kinase (DGK), an integral membrane protein from *Escherichia coli*. Twelve enhanced stability mutants of DGK were obtained using a simple screen. Four of the mutations were combined to create a quadruple mutant that had improved stability in a wide range of detergents. In n-octylglucoside, the wild-type DGK had a thermal inactivation half-life of 6 min at 55 °C, while the quadruple mutant displayed a half-life of 35 min at 80 °C. In addition, the quadruple mutant had improved thermodynamic stability. Our approach should be applicable to other membrane proteins that can be conveniently assayed.

Biochemical and structural studies of membrane proteins often require the proteins to be extracted from the lipid bilayer into a detergent micelle. Membrane proteins often exhibit unfavorable properties in detergent solution, however, such as poor solubility or stability (1, 2). Since the interactions between membrane proteins and detergents are very complex, the same protein can display different properties in different detergents. Thus, it is usually fruitful to screen for a detergent in which the protein of interest is active and stable. Unfortunately, many membrane proteins are only marginally stable and short-lived in even the best available detergent. In such cases, there are few alternatives for improving the experimental properties of the protein and the system can remain largely intractable.

Recently, we discovered that single side-chain alterations can significantly improve the properties of the membrane protein diacylglycerol kinase (DGK) from *Escherichia coli*, in detergent solution and that these stabilizing mutations can occur with high frequency. DGK is a 121-residue, trimeric enzyme, containing three transmembrane helices, that catalyzes the conversion of diacylglycerol and ATP to phosphatic acid (3–7). We examined the stability of 20 different cysteine substitution mutants in DGK and found two mutations (I53C and I70C) that significantly enhanced the resistance of DGK to thermal inactivation. These results suggested that it might not be difficult to identify stabilizing mutations in a pool of random mutants (11). Here we show that enhanced stability mutants of DGK can be readily identified by screening a mutant library and that a robust membrane protein can be constructed by combining individual mutations.

**EXPERIMENTAL PROCEDURES**

**Materials**—The detergents n-octyl-β-D-glucoside (OG), n-decyl-β-D-maltoside (DM), Cymal-5, and n-dodecyl-β-D-maltoside (DDM) were obtained from Anaspec. LDAO and Empigen were obtained from Calbiochem. Cardiolipin and 1,2-sn-dioleoylglycerol (DAG) were obtained from Avanti Polar Lipids. All other chemicals were from Fisher or Sigma.

**Mutagenesis**—We constructed a plasmid pSD005, containing a synthetic DGK gene. Plasmid pSD005 is identical to pSD004 (8), except that two mutations in the pSD004 sequence were modified to encode the natural E. coli sequence. Thus, pSD005 encodes an N-terminal half of DGK and 0.5% for the C-terminal half of DGK.

**Screening for Thermostable Mutants**—Mutagenized plasmids were transformed into an E. coli strain WH1061, in which the chromosomal DGK gene was disrupted (10). Individual colonies were inoculated into 600 μl of LB containing 100 μg/ml ampicillin and 50 μg/ml kanamycin in a 96-well plate format. After overnight growth with shaking at 37 °C, 4 μl of each culture were inoculated into 180 μl of fresh culture medium. Growth was monitored at 600 nm using a microplate reader with a path length correction function (Molecular Devices, SpectraMAX 340pc). All samples in the plate were induced by adding isopropyl-β-D-galactopyranoside to a final concentration of 200 μg/ml when the absorbance at 600 nm reached 0.8 for most of the samples. A 120-μl aliquot of each cell culture was then transferred into a new 96-well plate and the cells collected by centrifugation. The cells were resuspended in 120 μl of buffer containing 60 mM Pipes, 50 mM LiCl, 0.1 mM EDTA, 0.1 mM EGTA (pH 6.85), and 1 mg/ml lysozyme. To inactivate other kinases and ATPases, the cell lysate was transferred into a thin-wall 96-well PCR plate, and heated at 65 °C for 30 min in a thermocycler (MJ Research). DGK is highly resistant to this heat treatment in the intact membrane. To solubilize DGK, OG was added to a final concentration of 1.5% (w/v). Following the detergent extraction, a 30-μl aliquot of each sample was removed to a new plate and stored on ice. The remainder of the sample was heated at 55 °C for 10 or 15 min, followed by cooling on ice for 20 min. The enzyme activities of unheated samples (*A*<sub>0</sub>) and the residual activities of heated samples (*A*) were measured in 96-well plates as described (11). The remaining fractional activity (*A*/*A*<sub>0</sub>) was calculated for each sample. Samples satisfying the following two conditions were chosen as stable mutant candidates: (a) the remaining fractional activity was higher than the wild-type enzyme (*A*/*A*<sub>0</sub> > 1.5) and (b) the *A*<sub>0</sub> value was more than 10% of wild-type enzyme (*A*/*A*<sub>0</sub> × 10%). The candidates were tested again by the same screening method, and only those that passed the screen twice...
were sequenced and purified for further characterization.

**Enzyme Activity Assays**—Enzyme activities of both crude cell extracts and purified proteins were determined by a coupled enzyme assay system (8), in which the ADP generated by the DGR-catalyzed reaction was coupled to the oxidation of NADH. The transformation of NADH to NAD$^+$ can be monitored at 340 nm using a microplate reader (Molecular Devices, SpectraMAX 340PC), as described previously (11). Kinetic parameters were measured in the same manner, except the concentration of either MgATP or DAG was varied systematically.

**Protein Preparation**—Proteins were purified as described previously (8). Purified enzymes were stored at −80 °C in a buffer containing 50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, 0.25 mM imidazole, and 0.5% (w/v) DM.

**Thermal Inactivation Rates in OG**—Each enzyme was diluted 100 or 200-fold to a final concentration of 2–5 μg/ml in 50 mM sodium phosphate (pH 7.5), 0.3 M NaCl, and 1.5% (w/v) OG from a stock solution. Prior to measuring thermal inactivation rates, the enzymes were incubated on ice for at least 30 min, which was sometimes necessary to recover full activity after thawing the stored enzymes. 80-μl aliquots were heated in a thermocycler (MJ Research) at the desired temperature and then transferred to ice at given time intervals. The samples were incubated at room temperature for 10 min before their activities were assayed at room temperature as described above. The activity data could be fit well by a first-order rate equation.

**Thermostability in Different Detergents**—Each enzyme was diluted more than 100-fold to a final concentration of 2–5 μg/ml into 50 mM sodium phosphate (pH 7.5), 0.3 M NaCl, and one of the following detergents: 1.5% OG, 0.5% Empigen, 0.5% LDAO, 0.5% Cymal-5, 0.5% DM, 0.5% DDM. 80-μl aliquots were heated in a thermocycler at 45 °C, 55 °C, 65 °C, and 85 °C for 10 min, chilled on ice for 10 min, and then assayed at room temperature.

**SDS Denaturation**—SDS denaturation was carried out as described previously (8).

**Near UV Circular Dichroism Spectroscopy**—Near UV CD spectroscopy was performed using an Aviv model 62DS circular dichroism spectrometer. Proteins were exchanged into a buffer containing 10 mM Pipes (pH 7.0), 0.3 M NaCl, and 0.5% DM using a Sephadex G25M column (Amersham Pharmacia Biotech), and diluted to 0.46 mg/ml in the same buffer. Spectra were recorded in a 10-mm path length cell at 2-nm intervals using a 2-nm bandwidth and 1-s time constant. Twenty scans were averaged for each sample.

## RESULTS

**Identification of Thermostable Mutants**—A rapid screen, which could be carried out entirely in 96-well format plates, was developed to identify thermostable DGR variants from mutant libraries. A similar screen was used by Giver et al. to identify thermostable mutants of the soluble enzyme p-nitrobenzyl esterase (12). Individual clones were first grown and DGR expression induced in microtiter plates. After harvesting the cells, the DGR variants were solubilized in detergent. One aliquot of the extract was directly assayed for DGR activity, and a second aliquot was heated for 10 or 15 min at 55 °C. Clones that displayed total activity more than 10% of the wild-type enzyme activity, and a higher fractional activity after the heat treatment than the wild-type enzyme, were selected as thermostable candidates. The thermostable candidates were then validated first by re-screening in the same manner and then by measuring the thermal inactivation rates of the purified proteins.

A total of 1,559 colonies were screened, and 1,057 showed total activity more than 10% of the wild-type DGR activity before heat treatment. In the initial screen, 183 of the 1,057 active mutants were identified as thermostable candidates. Because our selection criterion was not particularly stringent (see “Experimental Procedures”), however, we expected a large number of false positives among the 183 candidates. The 183 candidates were therefore re-screened, reducing the number of thermostable candidates to 41. All 41 remaining colonies were sequenced, but only 18 possessed mutations in the DGR coding region. Thus, even after the second screen, a number of false positives remained. Nevertheless, we were still able to obtain a large number of thermostable mutants. As discussed below, all of the 18 mutants identified exhibited longer half-lives in detergent when purified.

Of the 18 mutants identified in our screen, 12 were distinct. The unique mutations are shown in Fig. 1. Three of the mutations (I53V, V107A, and W117G) occurred twice, and one mutation (E34V) occurred four times. Thus, we appear to have nearly saturated our ability to obtain mutants from these libraries after only screening 1,559 colonies. The 12 unique mutations occurred at eight positions in the DGR sequence. Most of the mutations were found at the edges of the putative transmembrane helices, except for positions 19 and 107, which reside in the middle of the first putative cytoplasmic helix and in the middle of the third putative transmembrane helix, respectively.

The thermal inactivation rates of the purified proteins were measured in the detergent OG. The results are shown in Table I along with the previously identified mutants, I53C and I70C. Although in many cases, the increases in half-life are modest, a number of the mutants had increases greater than 3-fold. The mutants I70L and I70C showed particularly dramatic increases in thermostability; no inactivation was observed after 40 min at 55 °C.

Although only mutants with a total activity more than 10% of the wild-type enzyme activity were selected in our screen, we did not correct for variations in expression level. We therefore measured the specific activity of the purified proteins (Fig. 2). All but one of the stable mutants have specific activities that were at least 50% of the wild-type enzyme. One mutation, I70L, even increases the specific activity by 50%. The least active mutant, E34V, still has 12% of the wild-type activity. These results indicate that the mutations did not destroy the ability of DGR to fold and function.
Combining Mutations with Enhanced Kinetic Stability—We attempted to improve stability even further by combining the most stabilizing substitutions. Four stable single mutants, I70L, V107D, I53C, and M96L were combined in the order of their effectiveness in stabilizing DGK. At each step of the combination, the thermal inactivation rate of the purified protein was measured at 80 °C (Table II). At this temperature, I70L was the only single mutant that displayed a measurable half-life, whereas V107D, I53C, and M96L were completely inactivated within 1 min of heat treatment (data not shown). As shown in Table II, each additional mutation adds to the half-life. The quadruple mutant, CLLD-DGK, showed a half-life of 35 min at 80 °C: almost 18 times that of the most stable single mutant and immeasurably better than the wild-type enzyme.

**CLLD-DGK Has Enhanced Stability in Different Detergents**—Because the screening experiments were carried out in the detergent OG, it is possible that the stabilization observed is specific to the OG environment. To test this possibility, we compared thermostability of wild type with CLLD-DGK in six different detergents that differ in both the apolar and polar groups. The purified enzymes were heated in different detergents for 10 min at temperatures ranging from 45 °C to 85 °C, and the residual activities after heat treatment were normalized with activities of unheated enzymes. As shown in Fig. 3, for all the detergents tested, the CLLD-DGK displayed higher residual activity compared with the wild type.

### Table I

**Half-life of DGK variants**

| DGK variant | Half-life at 55 °C | Increase in half-life |
|-------------|-------------------|----------------------|
| Wild type   | 5.7 ± 0.2         | NA                   |
| K19R        | 9.3 ± 0.7         | 1.6                  |
| E34V        | 14.9 ± 1.5        | 2.6                  |
| I53V        | 11.7 ± 0.3        | 2.1                  |
| I53T        | 11.9 ± 0.5        | 2.1                  |
| I53C⁺       | 28.7 ± 1.6        | 5.0                  |
| M66L        | 12.9 ± 1.5        | 2.3                  |
| M66V        | 18.6 ± 0.4        | 3.3                  |
| I70L        | —                 | —                    |
| I70T        | 10.2 ± 1.4        | 1.8                  |
| I70C⁺       | —                 | —                    |
| M96L        | 21.2 ± 1.3        | 3.7                  |
| V107D       | 33.0 ± 0.7        | 5.8                  |
| V107A       | 19.3 ± 0.5        | 3.4                  |
| W117G       | 14.0 ± 1.7        | 2.5                  |

* Mutants identified in previous work transferred into the pSD005 background (11).

**Combining mutants with enhanced stability**

| DGK variant | Amino acid positions | Half-life at 80 °C |
|-------------|----------------------|--------------------|
| Wild type   | 53 70 96 107         | <1 min             |
| Single mutant | I L M V            | 2                  |
| Double mutant | I L M D            | 5                  |
| Triple mutant | C L M D            | 11                 |
| Quadruple mutant | C L L D       | 35                 |

**TABLE II**

All half-lives reflect thermal inactivation rates of purified proteins in OG.

**CLLD-DGK Has Enhanced Thermodynamic Stability**—Improved kinetic stability can be partly a consequence of enhanced thermodynamic stability (13–15). We therefore compared the thermodynamic stability of CLLD-DGK with the wild-type protein using an assay we developed previously for DGK. In this assay, DGK unfolding is measured as a function of SDS concentration. Unfolding can be monitored by UV absorbance at 294 nm and by circular dichroism (CD) at 222 nm. When monitored by UV absorbance, two unfolding phases are observed. The first phase corresponds to an unfolding event involving the cytoplasmic domain and the second phase corresponds to the unfolding of membrane-embedded portion of DGK (8). CD only detects one phase, however, corresponding to the first transition seen by UV absorbance. The unfolding re-action is at or close to equilibrium throughout the observed transitions (8).

The SDS denaturation curves for the wild-type and CLLD-DGK are shown in Fig. 4. Little unfolding of CLLD-DGK is detected when unfolding is monitored either by UV absorbance or by CD, suggesting that CLLD-DGK is more resistant to denaturation by SDS than the wild-type enzyme. Thus, CLLD-DGK appears to be more thermodynamically stable than the wild-type enzyme. These results are supported by the fact that CLLD-DGK migrates as a stable trimer on SDS-PAGE, while the wild-type enzyme migrates as a monomer (data not shown). Thus, the mutations appear to enhance the stability of the oligomer. The fact that DGK longevity is increased at higher concentrations (data not shown) suggests a mechanism of irreversible inactivation involving a monomeric intermediate. The stabilizing mutations may therefore mitigate irreversible inactivation by decreasing the fraction of dissociated DGK.

**CLLD-DGK Displays Minimally Altered Structure and Catalytic Function**—While none of the individual mutations in CLLD-DGK inactivate the enzyme, it is important to verify that CLLD-DGK maintains a similar architecture to the wild-type enzyme and remains active. To investigate possible alterations in protein structure, the near UV CD spectrum of the quadruple mutant and the wild-type enzyme were compared (Fig. 5). Their nearly identical spectra indicate that they have similar structures. The structural integrity of CLLD-DGK was further confirmed, and the catalytic function assessed, by measuring kinetic parameters. As shown in Table III, the $K_m$ values for both ATP and DAG were very similar to the wild-type enzyme. Moreover, the $V_{max}$ value of the quadruple mutant was only 35% lower than the wild-type enzyme. Thus, stability enhancement has not come at the cost of enzyme function.
DISCUSSION

The results in this paper show that it is not difficult to identify mutations that improve longevity of the membrane protein DGK. In our case, about 1 in 87 colonies screened bore a mutant with increased stability. If the high frequency of stabilizing mutations translates to other systems, our approach may be applicable to any membrane protein that can be conveniently assayed. Thus, we now have a new weapon in our arsenal to attack a primary difficulty in working with membrane proteins: their poor stability in detergent micelles.

To use this approach on a wider spectrum of membrane proteins, including membrane proteins that are more difficult to assay, it would clearly be advantageous to learn what types of mutations are more likely to result in stabilization. If it were possible to target mutations more effectively, fewer mutants would need to be screened. In this light, it is interesting to note that six of the eight positions where stabilizing mutations occurred in DGK were found near the ends of the putative transmembrane helices. To determine if this is a general phenomenon or specific to DGK will require experiments with other membrane proteins. If the correlation holds up in others systems, however, future mutagenesis could be targeted to the regions that are near the membrane boundary.

More efficient engineering of stable membrane proteins may be aided by a greater understanding of the primary causes of inactivation, which are currently unknown. One obvious possible cause is that detergent does not mask the large hydrophobic surface of a membrane protein very effectively, making aggregation likely. If this were true, then strategies that seek to reduce the hydrophobicity of the transmembrane surface might be appropriate (16). We find, however, that mutations that do not alter hydrophobicity (such as I70L) can have dramatic effects on inactivation rates, suggesting that hydrophobicity is not a critical determinant of longevity.

Conformational flexibility of membrane proteins in detergent micelles may be an alternative explanation for their poor stability. Some conformations may be more susceptible to inactivation than others. It has been suggested that membrane protein structure is significantly influenced by ordering imposed by the bilayer (17–20). Thus, diminishing conformational restraints by extraction into a less ordered detergent micelle could increase conformational flexibility. If so, mutations that favor the less inactivation-prone conformations could slow irreversible inactivation. The fact that the CLLD-DGK mutant has enhanced thermodynamic stability suggests that increas-

![FIG. 3. Thermostability of wild type and CLLD-DGK in different detergents. The figure shows the fractional activity remaining after heat treatment for 10 min at the indicated temperatures. The results for the wild type are represented by the solid lines and for CLLD-DGK by the dashed lines. The different detergent environments are distinguished by the plot symbol as follows: OG (filled diamond), LDAO (filled circle), Empgen (open triangle), Cymal-5 (filled square), DM (open circle), DDM (filled triangle).]

![FIG. 4. Unfolding of the wild type and CLLD-DGK by SDS. A, unfolding monitored by absorbance at 294 nm. B, unfolding monitored by circular dichroism at 222 nm. The unfolding experiments were performed as described by Lau and Bowie (8). The absorbance and ellipticity values are normalized to range from 0 to 1.]

![FIG. 5. Circular dichroism spectra of DGK. Near UV CD spectra are shown for the wild type (filled circles) and CLLD-DGK (open triangles). Mean residue ellipticity is in units of degree cm² dmol⁻¹.

TABLE III
The kinetics parameters of wild-type and CLLD-DGK

|          | Km ATP | Km DAG | Vmax |
|----------|--------|--------|------|
| Wild-type| 0.24 ± 0.01 | 1.37 ± 0.02 | 87.4 ± 8.8 |
| CLLD-DGK| 0.18 ± 0.02 | 0.85 ± 0.03 | 56.9 ± 4.6 |
ing the stability of the folded conformation may indeed be a factor in determining detergent half-life.

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