Mapping the Oligomeric Interface of Diacylglycerol Kinase by Engineered Thiol Cross-Linking: Homologous Sites in the Transmembrane Domain†

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ABSTRACT: This work represents the first stage of thiol-based cross-linking studies to map the oligomeric interface of the homotrimeric membrane protein diacylglycerol kinase (DAGK). A total of 53 single-cysteine mutants spanning DAGK's three transmembrane segments and the first part of a cytoplasmic domain were purified and subjected to catalytic oxidation in mixed micelles. Four mutants (A52C, I53C, A74C, and I75C) were observed to undergo intratrimer disulfide bond formation between homologous sites on adjacent subunits. To establish whether the homologous sites are proximal in the ground-state conformation of DAGK or whether the disulfide bonds formed as a result of motions that brought normally distal sites into transient proximity, additional cross-linking experiments were carried out in three different milieus of varying fluidity [mixed micelles, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) vesicles, and *Escherichia coli* membranes]. Cross-linking experiments included disulfide bond formation under three different catalytic conditions [Cu(II)-phenanthroline oxidation, I_2 oxidation, and thionitrobenzoate-based thiol exchange] and reactions with a set of bifunctional thiol-reactive chemical cross-linkers presenting two different reactive chemistries and several spacer lengths. On the basis of these studies, residues 53 and 75 are judged to be in stable proximity within the DAGK homotrimer, while position 52 appears to be more distal and forms disulfide bonds only as a result of protein motions. Results for position 74 were ambiguous. In lipid vesicles and mixed micelles DAGK appears to execute motions that are not present in native membranes, with mobility also being higher for DAGK in mixed micelles than in POPC vesicles.

Prokaryotic diacylglycerol kinase (DAGK)[1] is an integral membrane protein located in the cytoplasmic membrane of many bacteria. It is a largely α-helical protein as determined by circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopy, with three transmembrane segments and two putative amphipathic helices near the membrane interface (Figure 1) (*1*, *2*). DAGK functions as a homotrimer of 13 kDa subunits (*3*) and catalyzes the direct phosphorylation of diacylglycerol (DAG) by MgATP (*4*). There are three active sites per trimer, which are known to lie at the interfaces between subunits (*5*). In Gram-negative bacteria, DAGK's primary function is believed to be as part of the membrane-derived oligosaccharide pathway (*6*), while in Gram-positive microorganisms, DAGK functions in the lipoteichoic acid biosynthetic pathway (*7*) and may also play a role in signal transduction (*8*).

As a membrane protein, DAGK has not to this point lent itself to structural determination by the techniques of solution NMR (*3*) or X-ray crystallography (J. U. Bowie, unpublished results). Therefore, nonclassical methodologies such as engineered thiol cross-linking are being pursued in order to obtain tertiary and quaternary structural information. Engineered thiol cross-linking involves the use of double cysteine mutants for monomeric proteins, while for oligomeric proteins, single-Cys mutants are typically employed. When exposed to oxidative conditions, pairs of thiols in these mutants may form disulfide-bonded products. When exposed to thiol-reactive bifunctional chemical cross-linking agents, pairs of thiols may be bridged with an intervening chemical spacer. For both classes of reactions, the rate and extent of cross-linking between thiol pairs will be, in part, a function of (1) proximity and relative geometry of reactive cysteine residues in the protein, (2) the frequency and amplitude of structural fluctuations of the protein, and (3) inherent reactivities of the SH groups in a particular environment. Cross-linking data can therefore provide information about both protein tertiary/

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[1] Abbreviations: BOG, *n*-octyl β-D-glucoside; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DBG, dibutyrylglycerol; DM, *n*-decyl β-D-maltoside; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPC, *n*-dodecylphosphocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; GA, glutaraldehyde; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; LDH, lactate dehydrogenase; MTS-2-MTS, 1,2-ethanediylbis(methanethiosulfonate); MTS-6-MTS, 1,6-hexanediylbis(methanethiosulfonate); NADH, nicotinamide adenine dinucleotide, reduced; NEM, *N*-ethylmaleimide; o-PDM, *N*,*N*′-*o*-phenylenedimaleimide; p-PDM, *N*,*N*′-*p*-phenylenedimaleimide; PIPES, 1,4-piperazinebis(ethanesulfonic acid); PK, pyruvate kinase; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TDS, 1-tetradecanesulfonic acid.

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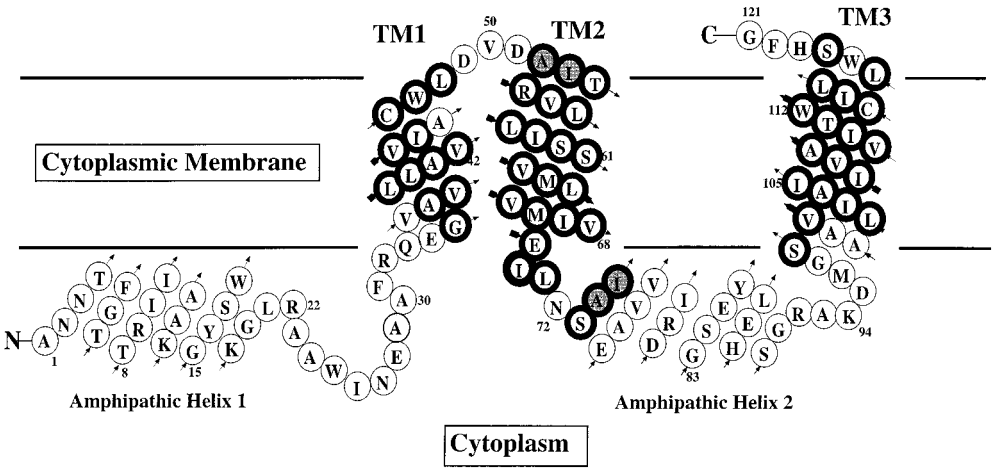

FIGURE 1: DAGK's membrane topology as determined by Smith et al. (*1*) and summary of Cu(II)-phenanthroline cross-linking results for DAGK in mixed micelles. Bold residues are those for which single-cysteine mutants were tested for homologous disulfide bond formation within the homotrimer. Shaded residues are those that were observed to form intratrimer disulfide bonds in the initial Cu(II)-phenanthroline screen of the homotrimeric mutants. Nonshaded residues did not form disulfide-bonded dimers. While DAGK is known to be highly helical (*2*), the exact beginnings and ends of its transmembrane helices are not known and the predicted amphipathic helices are strictly putative.

quaternary structure and protein motions. The engineered thiol cross-linking approach has been successfully employed as a tool for gaining information about membrane protein topology, elucidating the relative placement and orientation of transmembrane domains, and as a means of trapping conformations within dynamic proteins (*9*−*31*).

In this study, we present the results from the first stage of disulfide mapping studies of DAGK using a library of 53 single-cysteine DAGK mutants. The sites at which cysteine substitutions were made span most of the positions within DAGK's three transmembrane segments plus a few sites in a putative juxtamembrane loop that follows the second transmembrane segment (see Figure 1). The primary goals of this work were 2-fold. First, we wished to establish which sites, if any, within the transmembrane segments of one subunit are close to the corresponding sites on an adjacent subunit ("homologous sites"). For example, is the side chain for residue 40 on one subunit close to the side chain for residue 40 on another subunit within the homotrimer? Second, we sought to establish whether DAGK is generally a rigid or dynamic protein and whether its dynamics are environment-dependent.

MATERIALS AND METHODS

Preparation and Purification of DAGK Mutants. Polyhistidine-tagged DAGK single-cysteine mutants were constructed as described elsewhere (*2*). These mutants are based on a fully active "Cysless" mutant in which the two native cysteines of DAGK are replaced by alanine. Single-cysteine mutants were then generated by serially introducing a cysteine residue at each position in this Cysless mutant. The synthetic gene used for these studies is located on the plasmid pSD004 under *lac* repressor control for overproduction of polyhistidine tagged single-cysteine DAGK in *Escherichia coli* strain WH1061.

DAGK overproducing cells were grown at 37° C in Luria broth until OD_{600} reached 1.0 and then induced with IPTG, followed by 3 h of additional cell growth. Cells were harvested via centrifugation at 14000*g* for 15 min and lysed with lysozyme, DNase, and sonication as described elsewhere (*2*). Membrane proteins were normally solubilized by adding 3% octyl β-glucoside (BOG) (Anatrace, Maumee, OH) to lysate and mixing for 1 h at 4 °C. Insoluble material was removed by centrifugation at 27000*g* for 20 min. Solubilized protein was bound to Ni−NTA−agarose resin (Qiagen, Chatworth, CA) previously equilibrated with buffer A (0.3 M NaCl and 40 mM HEPES, pH 7.5) in the amount of 1.2 mL of resin for the extract from 1 g of cells. The resin was gently agitated for 1 h at 4 °C and then isolated via centrifugation at 1400*g* for 15 min. The resin was washed with 0.03 M imidazole and 1.5% BOG in buffer A, pH 7.8, to remove non-His-tagged proteins. The resin was then rinsed with 3 column volumes of 1% decyl β-maltoside (DM, Anatrace) in H_2O, and pure DAGK was eluted with 0.3 M imidazole and 1% DM, pH 7.8. In the case of DAGK destined for reconstitution into POPC vesicles, 1% *n*-dodecylphosphocholine (DPC, Anatrace) was used instead of DM in both the final rinse and elution steps. All purification steps were performed in cold buffers saturated with argon. Purified DAGK was routinely observed to be >95% pure by SDS−PAGE. DAGK's catalytic activity was routinely measured in mixed micelles or in lipid vesicles as described elsewhere (*4*, *33*).

Preparation of DAGK-Containing E. coli Membranes. E. coli lysate (see above, no detergent present) was centrifuged at 12000*g* for 20 min to remove unlysed cells and debris. The supernatant was then centrifuged at 125000*g* for 1 h. The resulting membrane pellet was washed/recentrifuged in 20 mM sodium phosphate, pH 7.0, 2 M KCl, 10% glycerol, 10 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM *o*-phenanthroline at 125000*g* for 1 h. The pellet was washed/recentrifuged two times in the above buffer with variations in the concentrations of DTT, KCl, *o*-phenanthroline, and EDTA (see ref *34*). The final membrane pellet was resuspended and stored at −70 °C until use.

Reconstitution of DAGK into POPC Vesicles. Purified DAGK in DPC micelles was mixed with a lipid stock solution of 0.2 M DPC and 50 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; Avanti Polar Lipids, Alabaster, AL) in 40 mM HEPES and 0.3 M NaCl, pH 8.0, such that the molar ratio of DAGK:POPC was 1:120. This

solution was added to 8000 molecular weight cutoff dialysis tubing (Spectra/Por 1.1) and dialyzed against 4 L of 10 mM imidazole, 0.5 mM EDTA, pH 7.8, and 0.2 mM DTT. Dialysis continued for 72 h at room temperature with two changes of buffer (>1000× volume) approximately every 24 h. Additional detail can be found in ref *33*.

Oxidative Disulfide Bond Formation of DAGK in Mixed Micelles, POPC Vesicles, and E. coli Membranes. Reactions involving the use of Cu(II) as the oxidation catalyst were carried out in several environments. For reactions in mixed micelles, purified DAGK was diluted to 14 μM into an "oxidation buffer" containing 20 mM sodium phosphate and 150 mM NaCl, pH 7.5, plus 1% (21 mM) DM and 2 mM tetradecanesulfonic acid (TDS, a lipidlike surfactant). Copper-phenanthroline was added to 1 mM from a freshly made 40 mM Cu(II) and 120 mM *o*-phenanthroline stock solution. Reactions proceeded at 4 °C in the presence of ambient oxygen and were then quenched by the addition of 10 mM EDTA to sequester Cu(II) and 10 mM *N*-ethylmaleimide (NEM) to block remaining unreacted SH groups. Products were analyzed via SDS−PAGE on 4−12% NuPAGE Bis-Tris gels (Novex, San Diego, CA) and the percent cross-linking was determined by integrating band density. In the case of POPC vesicular DAGK, reactions were carried out exactly as described above except in a detergent-free buffer.

DAGK in *E. coli* membranes (ca. 0.1−10 μM) was cross-linked in 20 mM sodium phosphate, pH 7.0, 10% glycerol, 0.1 mM EDTA, and 0.5 mM PMSF at 4 °C in the presence of 1 mM copper-phenanthroline. Reactions were quenched with 10 mM EDTA and 10 mM NEM, and products were analyzed via Western blots probed with a Ni−NTA−alkaline phosphatase conjugate (Qiagen) specific for DAGK's N-terminal polyHis tag.

Reactions involving the use of iodine to initiate disulfide bond formation were carried out as follows. For reactions in mixed micelles or POPC vesicles, DAGK was diluted to 14 μM in the same buffer as used for Cu(II)-catalyzed reactions. A 1 mM aqueous iodine stock solution was prepared by dilution of a 0.2 M iodine solution in ethanol. Aqueous iodine was then added (to 0.1 mM) to DAGK solutions and reactions were allowed to proceed at 4 °C for 1 min, followed by quenching with 10 mM NEM to block remaining unreacted SH groups. Iodine-catalyzed reactions in *E. coli* membranes were carried out at an effective DAGK concentration of 0.1−10 μM in 20 mM sodium phosphate, pH 7.0, 10% glycerol, 0.1 mM EDTA, and 0.5 mM PMSF. Iodine was added to 0.1 mM and reactions proceeded at 4 °C for 1 min, followed by quenching by the addition of 10 mM NEM. Products were analyzed by the same methods as used for Cu(II)-catalyzed reactions.

Double Cross-Linking of DAGK Single-Cysteine Mutants. To confirm that oxidatively induced disulfide bonds formed within homotrimers rather than between homotrimers, double cross-linking experiments were performed as follows. DAGK (14 μM) in DM/POPC mixed micelles was cross-linked in the presence of 1 mM Cu(II)-phenanthroline at 4 °C for 45 min, as described above. Reactions were quenched by the addition of 10 mM EDTA and 10 mM NEM. Glutaraldehyde (Sigma) was then added to 16 mM from a 25% aqueous stock solution. Samples were shaken vigorously for 16 h at room temperature and products were analyzed via SDS−PAGE on 4−12% NuPAGE Bis-Tris gels (Novex).

Forced Choice of Disulfide Bond Formation with DTNB. Vesicular DAGK was diluted to 14 μM in detergent-free buffer (40 mM HEPES and 0.3 M NaCl, pH 7.5). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was added to varying concentrations (0, 0.1, 0.5, 1.0, and 10 mM) from a fresh 25 mM stock in detergent-free buffer. Reactions proceeded at 4 °C for 20 min and were quenched by the addition of 10 mM NEM to protect any unreacted SH groups, and products were analyzed using SDS−PAGE.

Chemical Cross-Linking with Homobifunctional Reagents. Chemical cross-linking of DAGK was carried out in DM/POPC mixed micelles, POPC vesicles, and *E. coli* membranes with several reagents having two thiol-reactive moieties separated by a spacer of varying lengths, including *N*,*N*′-*p*-phenylenedimaleimide (p-PDM; Research Organics Inc., Cleveland, OH; 10 Å spacing between reactive moieties), *N*,*N*′-*o*-phenylenedimaleimide (o-PDM; Sigma; 6 Å), 1,2-ethanediylbis(methanethiosulfonate) (MTS-2-MTS; Toronto Research Chemicals, Toronto, ON; 7 Å spacing when fully extended), and 1,6-hexanediylbis(methanethiosulfonate) (MTS-6-MTS; Toronto Research Chemicals; 12 Å, fully extended). *o*-PDM and *p*-PDM were prepared as 25 mM stock solutions in dimethylformamide (DMF), while MTS-2-MTS and MTS-6-MTS were prepared as 0.1 M stock solutions in dimethyl sulfoxide (DMSO). Cross-linking reagents were added to 1 mM in DAGK-containing solutions, and reactions proceeded at 4 °C for 30 min. Reactions were carried out in mixed micelles (reconstituted DAGK diluted to 14 μM into a solution of 1% DM), in POPC vesicles (14 μM reconstituted DAGK in 40 mM HEPES and 0.3 M NaCl, pH 7.5), and in *E. coli* membranes (ca. 10−0.1 μM DAGK in 20 mM sodium phosphate, pH 7.0, 10% glycerol, 0.1 mM EDTA, and 0.5 mM PMSF). All reactions were quenched by addition of NEM to 10 mM to protect unreacted sulfhydryls, while PDM reactions were also quenched by the addition of 10 mM DTT.

RESULTS

Purification and Characterization of Single-Cysteine Mutants: Dealing with the Problem of Misfolding. The single-cysteine DAGK mutants that were examined in this work involve cysteine sites believed to lie within the three transmembrane segments of the enzyme plus sites in a putative juxtamembrane loop following the second transmembrane segment (Figure 1). Single-cysteine mutants were available for about 90% of the transmembrane positions. The goal was to examine cross-linking between identical sequential ("homologous") positions on adjacent subunits; studies were carried out with true homotrimers of single mutants. For the initial phase of this study (next section), we chose to carry out disulfide mapping in mixed micelles rather than in vesicles or *E. coli* membranes for two reasons: (1) use of micelles allows experiments involving the addition of cross-linking reagents to be carried out without concern that the compartmentalization which accompanies vesicles and *E. coli* membranes may prohibit ideal mixing of reagents, and (2) mixed micelles represent an environment in which protein motions are enhanced (see Discussion), such that disulfide bonds should form somewhat more freely than in the more constrained environment of vesicles or *E. coli* membranes. For the initial screen of mutants, it was preferred that disulfide bonds form too readily rather than not readily

enough. As will be seen, it is ultimately relatively straightforward to identify false positive cross-linking results, while false negatives (failure of truly proximal thiol pairs to form disulfide bonds) are much harder to identify.

Mutants were purified into detergent micelle solutions and assayed in mixed micelles for DAGK activity. For many mutants, activities at this stage were observed to be much lower than for wild-type DAGK. We have previously shown that such low activities for micelle-purified DAGK mutants are usually due to protein misfolding rather than active-site perturbations of correctly folded protein or thermodynamic instability of the mutants (*33*). Low-activity mutants were therefore routinely subjected to a "reconstitutive refolding" procedure (*33*). For most mutants this procedure leads to DAGK that exhibits high activity within its POPC vesicular host. What is more, when vesicular DAGK is redissolved back into micelles it typically exhibits dramatically enhanced mixed micellar catalytic activity compared to the prereconstitution activity. For example, for the four mutants of prime interest to this study (single cysteine at positions 52, 53, 74, and 75) the mixed micellar activities (given as percentages relative to wild type) before and after the refolding procedures were observed to be 10 ± 9 (before) to 64 ± 9 (after); 83 ± 12 to 100 ± 10; 3 ± 1 to 43 ± 10; and 2 ± 1 to 40 ± 10, respectively. In cases where the postrefolding activities were observed to remain well below that of wild type, it was not trivial to establish whether final lower-than-wild type activities represent a structurally nonperturbed active-site mutant or whether the misfolding process was less than 100% efficient. Certainly, activities for the A74C and I75C mutants are expected to be perturbed because these sites are highly proximal to DAGK's active site (*5*). In any case, for reduced activity mutants, key cross-linking experiments were carried out both before and after refolding. By comparing patterns before refolding (when misfolded protein dominated) to patterns after refolding (when the upper limit to the percentage of possible misfolded protein was in all cases much lower), it was possible to identify spurious cross-linking results. For example, by this procedure four single-cysteine mutants were identified (C58, C62, C65, and C104) that formed disulfide bonds under oxidative conditions in mixed micelles prior to the refolding procedure but did not form disulfide bonds in mixed micelles following reconstitutive refolding. In such cases, disulfide bond formation clearly reflects more about the structure of the misfolded protein than it does about correctly folded DAGK.

Cu(II)-Catalyzed Oxidation of DAGK Single-Cysteine Mutants in Mixed Micelles. We chose Cu(II)-phenanthroline (*9*, *11*, *35*, *36*) as the catalyst for the initial screening phase of these studies because it is relatively promiscuous in its promotion of disulfide bonds: it catalyzes S−S formation both between thiols that exist in stable proximity and between those that become proximal only occasionally as a result of protein motions (*11*, *21*).

In the case of single-cysteine DAGK mutants, cross-linking to completion within homotrimeric DAGK would lead to two out of three subunits being covalently linked by an S−S bond, with one subunit being left out. Since it is known that monomers are not free to exchange between homotrimers under the conditions of our experiments (Sanders, unpublished results), this would lead to an SDS−PAGE pattern in which $^2/_3$ of the DAGK would migrate as a dimer and $^1/_3$ would migrate as a monomer. However, disulfide bond formation under oxidative conditions is competitive with terminal oxidation of thiols to sulfonates ($R\text{-}SO_3^-$), such that complete 67% conversion in the case of DAGK mutants will rarely be observed (*11*, *36*, *37*).

Fifty-three homotrimeric single-cysteine mutants in mixed micelles were exposed to ambient oxygen in the presence and absence of 1 mM copper-phenanthroline catalyst. We observed several classes of results: mutants that did not form disulfide bonds, mutants that formed disulfide-bonded dimers only in the presence of catalyst, and mutants that formed disulfide-bonded dimers even in the absence of catalyst.

For those mutants observed to form dimers under any of the above conditions, we sought to establish whether disulfide bonds were forming between single subunits of different trimers (intertrimer dimerization) or whether they were forming within trimers (intratrimer dimerization). Therefore, the concentration of DAGK within a fixed concentration of micelles was decreased 100-fold and oxidative conditions were again introduced. Formation of intertrimer dimers was suppressed by this surface dilution procedure, thus decreasing the probability that trimers would come into contact.

Upon dilution, five homotrimeric mutants (C48, C52, C53, C74, and C75) continued to form disulfide-bonded dimers in the presence of catalyst, with C53 forming disulfide bonds even in the absence of catalyst. These mutants were subjected to an additional, more stringent, double cross-linking experiment to confirm that disulfide-bonded products were intratrimeric in nature. If disulfide-bond formation results from intertrimeric interactions, nonspecific glutaraldehyde (GA) cross-linking will trap the two disulfide-linked trimers resulting in a hexamer visible by SDS−PAGE. However, if disulfide bond formation is exclusively intratrimeric, GA cross-linking will trap no oligomers higher than trimer. Mutants C48, C52, C53, C74, and C75 were subjected to Cu(II)-phenanthroline oxidative conditions to induce disulfide bond formation, followed by nonspecific cross-linking with GA. In the absence of disulfide bonds, GA reacts with DAGK mutants to form covalent trimers with fairly high efficiency (*3*), as exemplified in Figure 2. In the cases of C52, C53, C74, and C75, when disulfide-bonded DAGK is cross-linked with GA, oligomers higher than trimers are not observed. For these sites, disulfide bond formation is clearly intratrimeric. However, in the case of C48, some hexamer is observed to form when the disulfide-bonded form is subjected to GA cross-linking. Thus, disulfide bond formation for site 48 appears to be at least partially intertrimeric in nature.

The four mutants (C52, C53, C74, and C75) that clearly formed intratrimer disulfide bonds can be classified into three groups on the basis of relative rates of disulfide bond formation. Positions 52 and 74 react relatively slowly, with the concentration of cross-linked product increasing as a function of time (Figure 3A). The fact that C74 yielded dimer in a somewhat lower yield than in the corresponding reaction represented in Figure 2 reflects batch-to-batch quantitative variability observed for this mutant, which most likely reflects the fact that the refolding of this mutant was not always optimal. In the case of C52, the low final yields observed may indicate that terminal oxidation of C52 thiols to sulfonates (see above and ref *11*) is highly competitive with disulfide formation; however, it could also reflect the

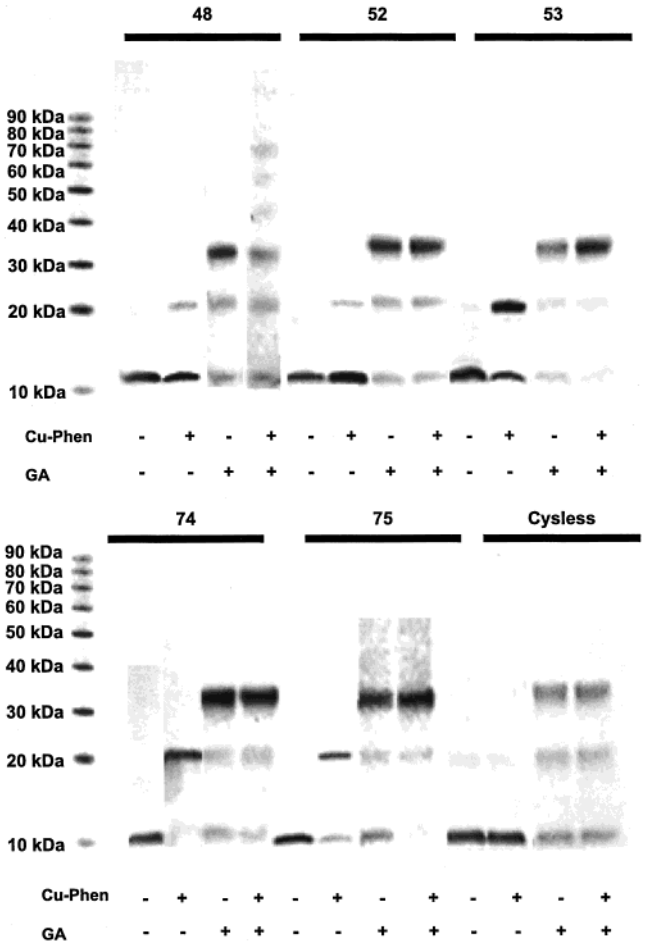

FIGURE 2: SDS−PAGE of single-cysteine mutants in DM/POPC mixed micelles following double cross-linking experiments in which mixed micellar DAGK mutants were subjected to copper-phenanthroline oxidation to promote disulfide bond formation followed by nonspecific GA cross-linking. Plus/minus signs indicate which reagent was present in the reaction represented in each lane. The reaction conditions are described in the Materials and Methods section but are essentially identical to those used for the samples of Figure 3. It should be noted that the monomer band in the Cu−phen-only lane for C74 is visible to the eye as a rather diffuse band on the original gel but did not reproduce well for this figure.

presence of some incompletely refolded DAGK that does not form disulfide bonds. Dimerization of C75 (to ca. 50%) is more rapid and complete within the first 5 min following copper-phenanthroline addition (Figure 3A). Position 53 readily forms disulfide bonds even in the absence of catalyst (see zero time point in Figure 3A). Following the addition of catalyst, dimer forms to >50% within 5 min (Figure 3A).

Cu(II)-Catalyzed Oxidation of DAGK Mutants in Bilayers and E. coli Membranes. The four single-cysteine DAGK mutants that were observed to form intratrimer disulfide-bonded dimers in mixed micelles were also cross-linked in POPC vesicles, where motions are expected to be more restricted than in mixed micelles. Vesicles represent a bilayered system having a single well-defined lipid composition. Under the conditions of our studies, the vesicles were in the fluid liquid crystalline phase (T_m −2 °C) (*38*). When catalytically cross-linked in POPC vesicles, positions 52, 53, and 75 all formed detectable dimers within 5 min of catalyst addition to a similar extent as in mixed micelles (Figure 3B). Position 74 formed little dimer in vesicles, unlike mixed micelles.

The four intratrimer disulfide bonding mutants (C52, C53, C74, and C75) were also cross-linked in isolated *E. coli* membranes (Figure 3C). As in POPC vesicles, C74 did not dimerize. C52 also failed to dimerize in *E. coli* membranes. However, following addition of Cu(II)-phenanthroline, C53 and C75 rapidly dimerized within *E. coli* membranes to ca. 25% and 50%, respectively (Figure 3C). The fact that C53 consistently did not dimerize in the presence of Cu(II) to as high a percentage in *E. coli* membranes as in vesicles (compare Figure 3 panels B and C) suggests either that the reaction partitioning ratio between sulfonate and disulfide bond formation may be different in *E. coli* membranes than in artificial vesicles (see Discussion) or that there may be additional fates available for C53 in *E. coli* membranes besides disulfide bond or sulfonate formation (e.g., modification of SH by products of lipid peroxidation).

"Forced Choice" of Disulfide Bond Formation. Results from the above sections suggest that positions 53 and 75 are highly proximal on homologous sites of DAGK subunits, while 52 and 74 may be somewhat less proximal and form disulfide bonds through motions. To confirm this interpretation, we carried out an experiment in which Cys-S−S-Cys formation was forced to compete with Cys-S−S-R formation where R is a small organic moiety. This was accomplished by exposing single-cysteine DAGK mutants to various levels of DTNB (*39*). The rationale of this experiment is that when the first thiol of any pair of homologous cysteines within a trimer reacts with DTNB, the second thiol will be forced to make a choice between attacking the neighboring thionitrobenzoate-activated cysteine thiol or reacting with exogenous DTNB. Because the leaving group (thionitrobenzoate) is the same regardless of which choice is made, the reaction partitioning ratio should ideally be a function of the proximity of the pair of cysteines and the DTNB concentration.

Single-cysteine mutants 52, 53, 74, and 75 in POPC vesicles were each exposed to four concentrations of DTNB ranging from 0.1 to 10 mM for a fixed period of time, followed by SDS−PAGE analysis. C52 and C74 failed to form dimers at any DTNB concentration (data not shown), consistent with low proximity in the "ground-state" conformation of DAGK. However, C53 and C75 both formed detectable dimer. In the case of C53, the yield of dimer was greater than 50% in the presence of DTNB from 1 to 10 mM, suggestive of high proximity for the homologous site in the homotrimer. Even at the highest DTNB concentration tested, thiol pairs prefer the pathway leading to subunit−subunit S−S bond formation rather than the pathway leading to two DAGK-S−S-TNB. In the case of position 75, the pathway leading to subunit−subunit S−S bond formation is less highly preferred (Figure 4), suggesting that pairs of Cys75 thiols are somewhat less proximal than those of C53.[2]

Iodine-Catalyzed Oxidation of DAGK Mutants in Mixed Micelles, POPC Vesicles, and E. coli Membranes. A third method of promoting disulfide bond formation was applied to mutants 52, 53, 74, and 75 by use of iodine as the oxidative catalyst. Thiol oxidation by iodine proceeds at

[2] It should be pointed out that the C75 sample used for acquiring the data of Figure 4 readily cross-linked in the presence of Cu(II)-phenanthroline to greater than 50% conversion to dimer. Thus, the much lower yield of DTNB-facilitated dimer for C75 relative to C53 cannot be explained by the possible presence of an unreactive misfolded C75 population.

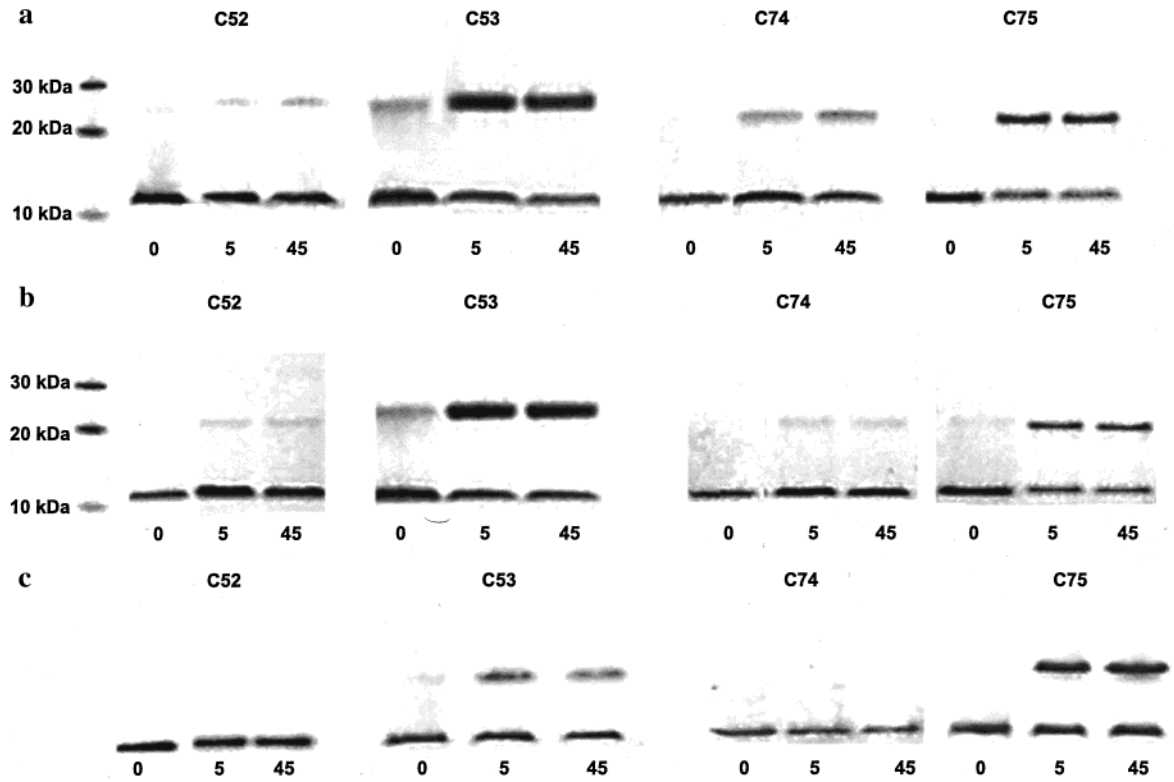

FIGURE 3: SDS−PAGE results for selected single-cysteine DAGK mutants following Cu(II)-catalyzed oxidation in (A) DM/POPC mixed micelles, (B) POPC vesicles, and (C) *E. coli* membranes. Reactions were carried out at 4 °C in the presence of 1 mM copper-phenanthroline. The effective DAGK concentration in mixed micelles and POPC vesicles was 14 μM, while the concentration in *E. coli* membranes was ca. 0.1−10 μM. The numbers under each gel are the reaction times in minutes. Additional technical details are given in the Materials and Methods section.

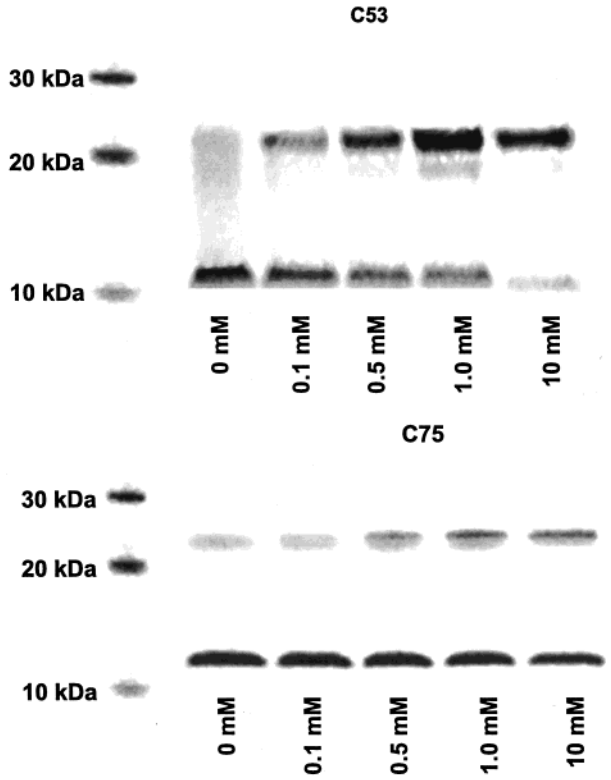

FIGURE 4: SDS−PAGE of selected DAGK mutants in POPC following "forced choice" of disulfide bond formation with DTNB in POPC vesicles. Vesicular DAGK (14 μM) was incubated at 4 °C in the presence of varying concentrations of DTNB as indicated for 20 min.[2] Additional technical details are given in the Materials and Methods section.

different rates and via a different chemical mechanism than Cu(II)-catalyzed oxidation (*21*, *36*, *37*). DAGK in mixed micelles, POPC vesicles, and *E. coli* membranes was exposed to 0.1 mM iodine and ambient oxygen to initiate the formation of disulfide-bonded dimers. Results are illustrated in Figure 5. Reactions carried out at higher iodine concentrations led to the formation of substantial dimers in most cases but were also observed to lead to full or partial destruction of DAGK through oxidative side reactions. Indeed, some destruction is evident even in the low-iodine cases illustrated in Figure 5.

C52 exhibited little tendency to form disulfide bonds with 0.1 mM iodine as an oxidative catalyst in micelles, vesicles, or *E. coli* membranes (Figure 5). C53 formed considerable dimer in all three membrane media tested. C75 formed dimers in all three media; however, the yield was much higher in *E. coli* membranes than in mixed micelles or vesicles. This may be due to the presence of some residual misfolded C75 DAGK in mixed micelles or vesicles that does not form disulfide bonds. In the case of C74, considerable dimer formed in the presence of iodine in mixed micelles and in POPC vesicles (Figure 5A,B). The yield of C74 dimer under these conditions was much higher than observed under conditions of Cu(II) catalysis (Figure 3A,B). This observation, combined with C74's failure to form dimers in the forced choice experiments, suggests two possibilities. First, the thiols at this position may approach each other only through the influence of motion, and for this position the iodine-based oxidative mechanism may be much more efficient at trapping the thiols in a disulfide bond than the Cu(II) mechanism. A second possible interpretation is that

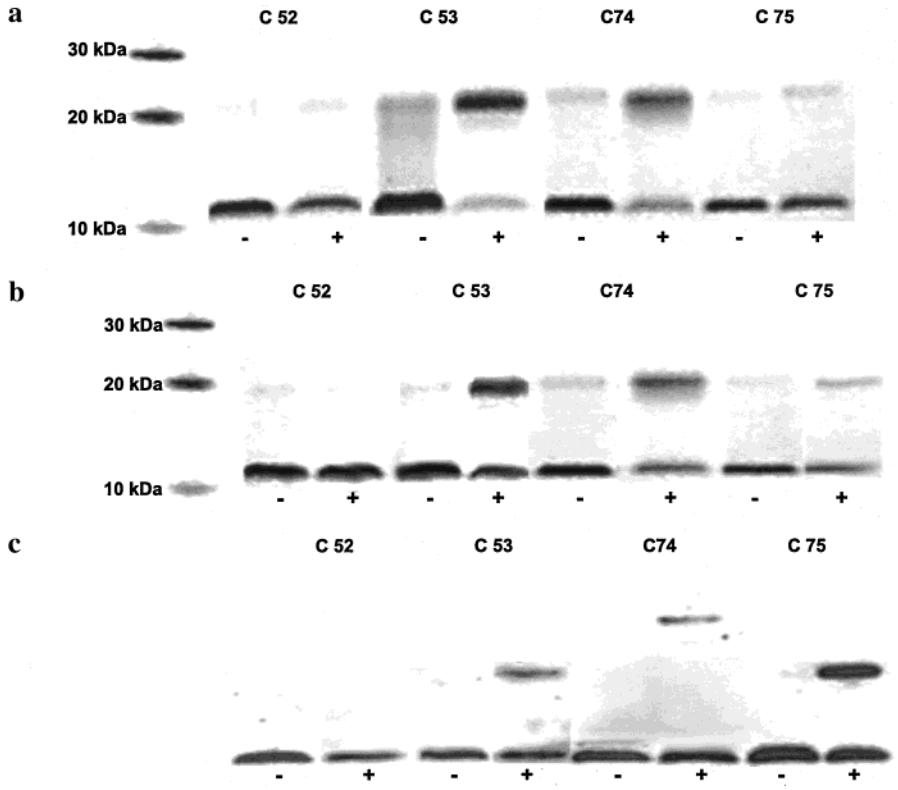

FIGURE 5: SDS−PAGE following iodine-catalyzed cross-linking of DAGK single-cysteine mutants in (A) DM/POPC mixed micelles, (B) POPC vesicles, and (C) *E. coli* membranes. The effective DAGK concentration in mixed micelles and POPC vesicles was 14 μM, while the concentration in *E. coli* membranes was ca. 0.1−10 μM. DAGK was incubated in the presence of 0.1 mM iodine at 4 °C. Reactions were quenched with 10 mM NEM 1 min after addition of catalyst. (−) No I_2 added; (+) 0.1 mM I_2 present. Additional technical details are given in the Materials and Methods section.

C74 is indeed homologously proximal but that for some reason(s) disulfide bond formation under the influence of Cu(II)-phenanthroline and thionitrobenzoate catalysis is unusually inefficient. In *E. coli* membranes dimeric C74 did not appear as a result of iodine exposure, but a band that may represent trimeric DAGK appeared (Figure 5C). The fact that higher concentrations of iodine appear to completely destroy DAGK (alluded to above) suggests that this result probably reflects iodine's ability to catalyze other protein-involved reactions in addition to thiol oxidation.

Estimating Proximity with Homobifunctional Cross-Linking Reagents. We next examined the reactions of mutants C52, C53, C74, and C75 with bifunctional thiol-reactive cross-linking agents. Two pairs of reagents were employed, which contained moieties of varying lengths between thiol-reactive groups. The first pair involves maleimide groups separated by rigid spacers (p-PDM, 10 Å between reactive sites, and o-PDM, 6 Å). The second pair of reagents involves activated bisthiols separated by flexible linkers: MTS-2-MTS (7 Å when fully extended) and MTS-6-MTS (12 Å).

When cross-linkers were added to mixed micelles and POPC vesicles, positions 52, 53, 74, and 75 reacted with at least one reagent each (Figure 6A,B). Cross-linkers were also added to *E. coli* membranes, where motions are reduced compared to vesicles. No cross-linking was observed with any cross-linking reagent for positions 52 and 74 in *E. coli* membranes. Data for positions 53 and 75 in *E. coli* membranes are shown in Figure 6C.

Position 53 reacted with all four reagents in mixed micelles and reconstituted vesicles, suggesting that while this residue is in high proximity within the ground-state conformation of the DAGK trimer, it is subject to motional excursion in micelles and artificial vesicles such that it can react even with widely spaced reactive moieties of rigid cross-linking reagents (up to at least 10 Å). The failure of C53 to react with the rigidly spaced PDM reagents in *E. coli* membranes may reflect high steric inaccessibility, reduced intrinsic maleimide reactivity near position 53 in *E. coli* membranes, or reduced motion relative to POPC vesicles. The fact that no leftover monomer band was observed in the case of C53 following reaction with MTS-2-MTS most likely results from anomalous migration of the modified monomer on the SDS−PAGE gel.[3] Position 75 reacted in both membranes and vesicles with a profile very similar to that of C53. Position 52 reacted strongly with MTS-2-MTS (>50%) in both mixed micelles and vesicles and to a lesser extent with o- and p-PDM. Position 74 did not react strongly with the PDM reagents under any conditions but reacted strongly (ca. 60%) with MTS-6-MTS in mixed micelles. Neither position 52 nor 74 reproducibly reacted with any cross-linking reagent in membranes.

Effect of Disulfide Cross-Linking on Enzymatic Activity. DAGK activities were measured for C52, C53, C74, and C75 under various cross-linking conditions.[4] For the C53 mutant, the percentage of original activity following cross-linking was as follows: Cu(II)-phenanthroline oxidation, 106%;

[3] On the basis of double copper-phenanthroline/GA cross-linking experiments on this MTS-2-MTS-modified C53 (see Materials and Methods for procedure) and unpublished subunit exchange studies, the "missing" monomer cannot be the result of reactions resulting from intertrimer cross-linking or from free monomeric exchange between subunits (which is extremely slow).

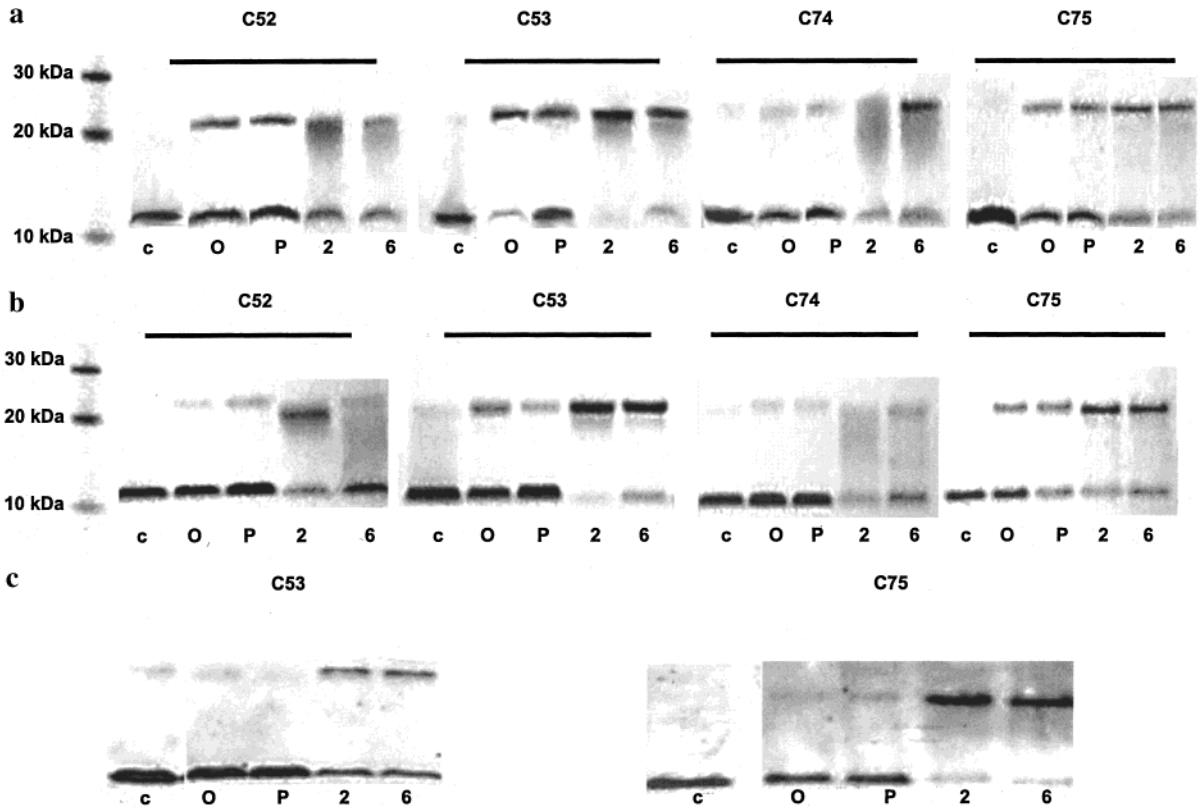

FIGURE 6: SDS−PAGE following cross-linking of single-Cys DAGK mutants with homobifunctional reagents in (A) DM/POPC mixed micelles, (B) POPC vesicles, and (C) *E. coli* membranes. DAGK was incubated in the presence of 1 mM of each cross-linker at 4 °C. Reactions were quenched after 30 min with 10 mM NEM. PDM reactions were also quenched with 10 mM DTT. c, no cross-linker present; P, *N*,*N*′-*p*-phenylenedimaleimide (10 Å spacer); O, *N*,*N*′-*o*-phenylenedimaleimide (6 Å); 2, 1,2-ethanediylbis(methanethiosulfonate) (7 Å when spacer is fully extended); 6, (1,6-hexanediylbis(methanethiosulfonate) (12 Å, fully extended). Additional technical details are presented in the Materials and Methods section.

cross-linking with o-PDM, 120%; cross-linking with p-PDM, 127%. For the C52 mutant, percentages of original activity were: Cu(II)-phenanthroline oxidation, 140%; cross-linking with o-PDM, 85%; cross-linking with p-PDM, 100%. In all cases, changes in activity are modest. These results do not shed light upon residue proximity, but they indicate that DAGK catalytic efficiency is largely independent of the imposition of fixed distances between pairs of position 52 and 53 thiols spanning a range of about 2−10 Å.

The activities of C74 and C75 mutants were in each case reduced by at least a factor of 10 following Cu(II)-phenanthroline oxidation or reactions with o-PDM and p-PDM.[4] These results are not too surprising and are not very informative because both positions are in or near the active site of the enzyme (*1*, *5*, *40*). These observed activity losses may reflect direct perturbations of the active site that are not necessarily related to perturbing DAGK dynamics or imposing fixed distances between residue pairs.

DISCUSSION

Thiol Cross-Linking as an Approach to Dynamic and Structural Measurements for DAGK. This study represents the first step in thiol-based cross-linking studies of DAGK. Quantifying intermolecular distances by chemical cross-linking is very difficult because a number of variables influence both the rate and maximum extent of any given cross-linking reaction at any given pair of sites (*11*, *20*, *21*, *27*). Moreover, these variables will change from reaction type to reaction type and from reagent to reagent. Rather than focusing upon careful quantitation of results of one or two reaction types (as very carefully done in ref *11*), we carried out a battery of more qualitative cross-linking reactions on each key mutant. Three different catalytic conditions for disulfide bond formation were used [Cu(II), I_2, and thionitrobenzoate-assisted). Bifunctional chemical cross-linking reagents were also employed in which spacer lengths, spacer flexibility, and reaction chemistry (thiol−disulfide exchange vs thiol attack of an electrophile) were varied. Furthermore, we also varied the overall dynamics of DAGK by varying the amphiphilic environment in which reactions were carried out (mixed micelles, artificial vesicles, and *E. coli* membranes). The rationale was that if a series of consistent results was obtained for a given mutant throughout the full series of reaction types tested, then it should be possible to establish

[4] Results from before and after iodine oxidation are not described in this section because iodine in all cases tested induced a large loss of activity, which reflects covalent damage of DAGK catalyzed by iodine that extends well beyond thiol oxidation. Cu(II)-phenanthroline is clearly a "cleaner" thiol oxidant than is iodine, at least in the case of DAGK, both in permitting DAGK activity to be retained and in avoiding destruction of the protein. Activities were also measured for these four residues following modification with the two MTS reagents. In the cases of C53, C74, and C75, activities following cross-linking were qualitatively similar to results for the PDM reagents. In the case of C52, activities following reaction with either MTS reagent were considerably lower than for the PDM reagents, for reasons that are not obvious. We consider the PDM results (no significant change in activity following reaction with these rigid cross-linkers) to be more telling than the MTS results since the reductions in activity observed for the latter case could reflect side reactions or other artifactual phenomena.

within an acceptably high probability the approximate ground-state distances between reactive thiol moieties and the amplitudes of motions that may bring normally distal pairs into close proximity.

On the basis of this general approach and the results presented in this paper, it appears that the β-carbons of position 53 are within 5 Å of each other on homologous sites within the homotrimer. Unless great care is taken, cysteines at this position form disulfide bonds even in the absence of an oxidative catalyst. Not only is disulfide bond formation rapid and efficient for this position under multiple conditions of oxidative catalysis, but efficiency is high when this position is forced to make a rapid choice between Cys-S−S-Cys formation and non-cross-linked products in reactions catalyzed by iodine or thionitrobenzoate. Moreover, when a C53 pair forms a disulfide bond within a DAGK homotrimer, catalytic activity is not significantly perturbed. However, while position 53 is proximal in the ground-state structure, DAGK is sufficiently dynamic (particularly in mixed micelles and artificial vesicles) such that cysteines at position 53 readily cross-link with bifunctional reagents having extended spacers as long as 12 Å.

It appears that the β-carbons at position 75 are within <6 Å of each other on homologous sites within the homotrimer. Reaction profiles of position 75 with copper, iodine, and DTNB are similar to C53 except that the yield or rate of formation of disulfide-bonded dimer is sometimes lower for position 75 and spontaneous disulfide bonds do not form in the absence of catalyst for this position. These results indicate that position 75 may not be quite as close to the corresponding position within the homotrimer as position 53. Unlike position 53, disulfide bond formation at 75 perturbs DAGK such that enzymatic activity is abolished. However, position 75 is similar to 53 in that it reacts with rigid bifunctional reagents with spacers as long as 10 Å in mixed micelles or vesicles, suggesting that while 75 is proximal in the ground state, it is still subject to large amplitude dynamics, at least in these model membrane media.

The various cross-linking results also indicate that position 52 undergoes motions appropriate to allow the detectable formation of disulfide bonds in mixed micelles and POPC vesicles under conditions of Cu(II)-catalyzed oxidation. However, disulfide bonds do not form under conditions of Cu(II)-catalyzed oxidation in *E. coli* membranes or under conditions of low iodine concentration in any medium. Also, the extent of disulfide bond formation in mixed micelles or POPC vesicles for position 52 is very low under "rapid forced choice" conditions imposed by thionitrobenzoate disulfide exchange. Thus, position 52 does not appear to be in high proximity within the dominant ground-state conformer(s) of DAGK.

Position 74 is the most difficult position for which to make a conclusion. In some experiments this position behaved like the distal site 52. However, in the presence of iodine it exhibited a high tendency to form disulfide bonds in mixed micelles and POPC vesicles. Overall, results for position 74 do not allow us to establish with certainty whether it is highly proximal.

Implications for DAGK's Three-Dimensional Structure. While DAGK's membrane topology, oligomeric state, and general secondary structure have been established (*1*−*3*) and while it is known that DAGK's active sites (three per trimer) lie at the interfaces between the monomers (*5*), little is known about its detailed tertiary or quaternary structure. This work represents an important step in the development of an experimentally based structural model. While about 90% of all of the sites within the three transmembrane segments of DAGK were tested for homologous disulfide bond formation within the DAGK trimer, only position 53 was clearly in high proximity. A second site, position 75, was also identified to be highly proximal, but it is not believed to lie within the transmembrane domain (see Figure 1). Position 53 represents the third position in a highly conserved motif in DAGK where positions 1 and 4 are almost always a pair of residues, one side chain of which can serve as a hydrogen-bond donor (usually S, T, Q, or N) while the other can serve as a hydrogen-bond acceptor (usually E, D, N, or Q). Position 2 is highly variable, but position 3 is usually a large hydrophobic residue. Such a motif (*41*, *42*) strongly suggests that these residues represent the "cap" or initiation site of transmembrane helix 2 (see Figure 1). The location of position 53 within this motif indicates that the N-terminus of this segment is a homotrimeric contact that must be located at the axis of 3-fold symmetry. Examples of high-resolution protein structures exist in which the β-carbon of side chains of particular homologous sites located at the axis of 3-fold symmetry of homotrimers are close enough (ca. 5 Å; see ref *11*) to easily form disulfide bonds (cf. *43*, *44*). Modeling of trimeric helix bundles for DAGK's TM2 (not shown) indicates that the failure to observe disulfide bond formation between homologous sites in the middle or C-terminus of TM2 implies either that (A) the central TM2 helices in a trimer are splayed outward from each other toward the cytoplasmic face, (B) the TM2 helices both tilt and twist in a helical bundle such that while C53 is close enough on the N-terminal side to cross-link, residues immediately above it on the same helical face (C60, C67) are not positioned toward each other in the helical bundle, or (C) the trimeric interface at the center of the membrane is so densely packed that the reactive oxygen species generated by copper-phenanthroline cannot access these sites. Additional study will be required to distinguish between these possibilities.

Position 75 was also observed to be highly proximal within the DAGK homotrimer, indicating that it too is near the axis of 3-fold symmetry. This residue lies within the third position of another highly conserved four-residue (SXXE) motif that may function as the N-terminal initiation site (*41*, *42*) of a putative cytoplasmic amphipathic helix. Alternately, it is possible that TM2 does not terminate near residue 70 as suggested in Figure 1 but extends further and includes position 75. C75 is highly proximal to residues believed to be involved in DAGK active sites, which Bowie and coworkers have recently demonstrated (*5*) to be "shared sites" involving residues from adjacent monomeric subunits (rather than each of the three active sites lying exclusively within a single subunit). Indeed, results from Bowie's study are consistent with homologous proximity of C75. In that study, subunits from inactive DAGK homotrimeric site 72 and site 76 mutants were mixed to form heterotrimers in which catalytic viability was observed to be partially restored, suggesting some degree of proximity for residues 72 and 76 on adjacent subunits. This observation, combined with the results for C75 in this paper, indicates that at least part of each of the three active sites of DAGK must also lie near the axis of 3-fold symmetry.

Lack of disulfide bond formation in the first and third transmembrane segments of DAGK coupled with the conclusion that TM2 forms the central axis of the trimer indicates that TM1 and TM3 must lie well away from the axis of 3-fold symmetry, probably forming an annulus of 6 helices that lie between the central three TM2 helices and the surrounding lipid bilayer.

DAGK Exhibits Environment-Dependent Motions. We observed that even in bilayered vesicles positions 53 and 75 undergo motions that lead to variation of interresidue distances between homologous sites on the order of at least 7 Å based on cross-linking with rigid bifunctional reagents. However, these same sites did not react with those reagents in *E. coli* membranes. Positions 52 and 74 did not usually form disulfide bonds in *E. coli* membranes but did form disulfide bonds in mixed micelles. In artificial POPC vesicles and under conditions of Cu(II) oxidation, position 52 formed disulfide bonds to about the same extent as in mixed micelles, while position 74 did not. Both 52 and 74 showed reduced reactivity with homobifunctional reagents in vesicles relative to mixed micelles and no reactivity at all in native membranes. In sum, these observations suggest that *E. coli* membranes are a relatively restrictive environment for DAGK compared to POPC vesicles which, in turn, are much more restrictive than mixed micelles. It has previously been demonstrated that lactose permease is also more flexible in micelles than in vesicles or *E. coli* membranes (*45*, *47*, *48*). Enhanced motions for DAGK in mixed micelles may help to account for difficulties in crystallizing this protein despite much effort (J. Bowie, unpublished results) and may also be a source of undesired conformational exchange-based line broadening in solution NMR spectra of micellar DAGK (*3*). The fact that only a few homologous sites in the transmembrane domain of DAGK formed disulfide bonds suggests that the motions present in micelles and mixed micelles may be fairly local (otherwise, more normally distal sites would have been expected to form disulfide bonds as a result of motions).

Restraining the motions involving positions 52 and 53 by forming cross-links of varying lengths between pairs of homologous sites was not observed to have a dramatic impact upon catalytic activity. This suggests that the motions which allow C52 and C53 thiol pairs to both form disulfide bonds and cross-link with bridging spacers of considerable length are not functionally critical. Moreover, DAGK's catalytic activity is rather insensitive to the imposition of a variety of distances between these sites. These observations may not be too surprising since the membrane−water interface at which these residues are located is on the opposite side of the membrane from the active site. In the case of positions 74 and 75, the intimate location of these residues near the active site makes it difficult to ascertain whether activity losses observed when these residues were cross-linked were due to motional modulation or to some other perturbation of the active site. Additional studies will be required to see whether some DAGK motions are related to the conformational changes typically observed for water-soluble kinases as a result of substrate binding (*46*).

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