Thiol modification of diacylglycerol kinase: dependence upon site membrane disposition and reagent hydrophobicity

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Abstract Reaction rates were determined between disulfide reagents of varying hydrophobicity and single-cysteine mutants of diacylglycerol kinase, an integral membrane protein. Polar reagents reacted most rapidly with surface-exposed sites. However, a very non-polar reagent also reacted more rapidly with exposed cysteines than with membrane sites. Moreover, this non-polar reagent usually reacted more slowly with membrane sites than did more polar reagents. These results are consistent with the notion that disulfide exchange reactions involving buried cysteines of diacylglycerol kinase are very slow in the membrane interior, such that the competing rates of reactions which occur when normally buried cysteine sites make motional excursions to hydrated regions of the interface can be significant.

Key words: Cysteine modification; Thiol; Membrane protein; Diacylglycerol kinase; Structure

1. Introduction

Integral membrane proteins represent an especially difficult target for structural analysis because of well-known difficulties in applying X-ray diffraction and solution NMR methods to proteins of this class. For this reason there has been particular interest in developing alternate routes to structural determination for membrane proteins. One class of approaches relies upon the introduction of cysteine residues at specific sites, followed by chemical or spectroscopic experiments which probe local structural questions for these sites [1–3]. For example, the rates of reactions of single-cysteine mutants with thiol-specific chemical modification reagents provide insight into site accessibility [4–6].

In this paper, we examine the use of cysteine modification rates to provide structural information. For any given cysteine located in a membrane protein the rate of reaction with a specific disulfide reagent under a given set of conditions can be expected to be a function of at least three factors. First, there will be a critical dependency upon site disposition with respect to the membrane. Polar disulfide reagents are expected to react more rapidly with cysteine sites located in water-exposed domains than with Cys in transmembrane segments. Secondly, reactivity will normally be reduced for sites which are buried in the interior of the protein relative to sites which are exposed to lipid or water. Third, reactivity will be a function of the pK_a of the reactive Cys thiol. This will be determined not only by the degree of water exposure, but also by the detailed local electrostatic/hydrogen bonding properties of any given site. Because there are at least three general variables which determine the reactivity of a single reagent with a protein site it is generally not possible to use a *single* kinetic measurement to establish structural information regarding membrane location and/or residue disposition at a protein–protein or a protein–solvent/lipid interface.

Given the above considerations, we reasoned that it might be possible to directly assess the disposition of any given protein site with respect to the membrane interface by measuring site reaction rates with a *series* of chemically-related reagents in which hydrophobicity is systematically varied. Naively, we expected hydrophobic reagents to show a preference for cysteine sites located within the membrane. In this paper, it is shown that this expectation was not always justified in the case of diacylglycerol kinase.

DAGK is a homotrimeric protein, with each subunit having an experimentally-determined membrane topology as illustrated in Fig. 1 [7,8]. Choice of DAGK for these studies was based upon three factors. First, a set of single-cysteine mutants of DAGK is available (James Bowie, unpublished), which permits systematic examination of the dependence of thiol reaction rates as a function of sequence position. A second motivating factor for using DAGK is the body of structural data already available for this protein. While DAGK's high resolution structure has yet to be determined, it is known that transmembrane helix 2 lies at the central 3-fold symmetry axis of the homotrimer [9]. It therefore follows that transmembrane segments 1 and/or 3 must lie on the periphery of the transmembrane bundle of helices in the homotrimer. This is supported by mutagenesis data showing that sites in TM2 are generally more highly conserved that sites in TM1 and TM3 [10]. By examining sites representing a complete turn on each of these two helices, it should be possible to sample transmembrane sites which have side chains which are either lipid exposed or facing the interior of the protein. The sites chosen for specific examination are indicated in Fig. 1. Finally, choice of DAGK for these studies is justified based on the fact that DAGK has a readily assayable catalytic activity which allowed us to probe and confirm the functional viability of the samples used in this study.

2. Materials and methods

2.1. Thiol modification reagents

Ellman's reagent (DTNB), 5,5′-dithio-bis(2-nitrobenzoic acid), was purchased from Sigma (St. Louis, MO, USA).

5-(1-Octanedithio)-2-nitrobenzoic acid (OTNB) was synthesized by reacting DTNB with 1-octanethiol. It was purified from side products and unreacted reagents using flash chromatography. The desired product was verified by proton NMR and thin layer chromatography.

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A similar approach was used to prepare 11-(2-pyridyldithio)-undecanoic acid (PTUND) following the reaction of 11-thioundecanoic acid with 2,2′-dithio-bis-pyridine. Full details of the syntheses of both OTNB and PTUND are available upon request from the authors.

2.2. Preparation of single-cysteine diacylglycerol kinase (DAGK)

Strains harboring mutants of *Escherichia coli* DAGK [11], each containing only a single cysteine residue were provided by the laboratory of James Bowie (UCLA). DAGK mutants were purified and reconstituted into 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, Avanti, Alabaster, AL, USA) vesicles by a procedure which is known to induce correct folding of the often misfolded enzyme [12]. The vesicular DAGK was then redissolved into decyl maltoside (DM, Anatrace, Maumee, OH, USA) solutions to yield correctly folded DAGK in DM–POPC mixed micelles which were used for chemical modification studies. The final DAGK:POPC mole:mole ratio in these samples was 1:120 and the mole fraction of POPC in the mixed micelles was 15%. For thiol exchange reactions the buffer was 100 mM sodium phosphate, 0.1 mM EDTA, pH 6.5. It should be pointed out that DAGK is fully catalytically functional under these mixed micellar conditions. DAGK samples used in this work were routinely assayed using a standard procedure [13] to confirm catalytic viability.

2.3. Modification of DAGK with thiol-specific reagents

0.07 mM (1 mg/ml) solutions of single-cysteine DAGK mutants in DM/POPC mixed micelles were incubated in 1 cm cuvettes at 30°C in a Hewlett-Packard model 8452 UV spectrophotometer. To these solutions were added stock solutions of DTNB (18 mM in reaction buffer), OTNB (55 mM in DMSO), or PTUND (55 mM in DMSO) such that the final concentration of the thiol-reactive reagent was in each case 0.33 mM. The reaction between the cysteine thiols of DAGK and the added reagent was monitored by light absorbance at 412 nm (DTNB and OTNB) or 343 nm (PTUND). The concentration of the chromophore generated during each reaction could be quantitated as a function of time using 1 cm extinction coefficients of 13.6 absorbance units per mM for thionitrobenzoic acid [14] and 8.0 absorbance units per mM for thiopyridine [15] at the wavelengths given above. Because all three reagents showed significant hydrolysis over a period of hours in DAGK/thiol-free solutions, 'blank' reactions were monitored so as to be able to correct reaction progress curves obtained for DAGK samples.

DAGK is homotrimeric and so there is the possibility of cooperativity in modification rates as the first, second, and third Cys within a homotrimer are successively modified. To avoid having to treat such cooperativity directly in measuring reaction rates, we focused upon measuring initial (linear) rates from the earliest part of each reaction progress curve. Thus, initial rates reflect reaction of the disulfide reagents with the first (of three) cysteines present in each DAGK homotrimer.

3. Results

3.1. Thiol modification reagents

The three compounds with which this paper is concerned are illustrated in Fig. 2. Three of these compounds have been used in previous studies [16,17], while PTUND is, to our knowledge, a novel reagent. All three compounds have activated disulfide bonds which are primed for efficient thiol–disulfide exchange reactions by virtue of the stability of the aromatic thiolate reaction product (thiopyridine or thionitrobenzoate). In this regard, these compounds are chemically analogous. The chromophoric nature of the aromatic thiolate products makes it simple to follow the time course of thiol exchange reactions.

The predicted membrane location of the reactive disulfide bond for each of the three reagents of Fig. 2 varies. DTNB is polar and freely water soluble and is not predicted to partition significantly into lipid bilayers or micelles. The other two compounds have apolar tails and are expected to be membrane associated. For OTNB, the TNB moiety forms the water-exposed head group, while the disulfide group is predicted to be located just into the membrane interface with the octyl tail extending into the hydrophobic interior. Disulfide bonds are rather apolar because of the 'soft' Lewis base nature of the lone pairs of electrons in the participating sulfur atoms. In the case of PTUND, the dithiopyridine moiety is located on the other end of the alkyl chain from its polar carboxylate head group. It can be calculated [18] that the dithiopyridine moiety by itself will have a strong preference for membranes relative to an aqueous phase. These same calculations also predict a slight (ca. 1 kcal/mol) preference for the water-saturated interfacial region of the membrane compared to the hydrocarbon interior. However, in the chemical context of the attached undecylcarboxylate chain of PTUND,

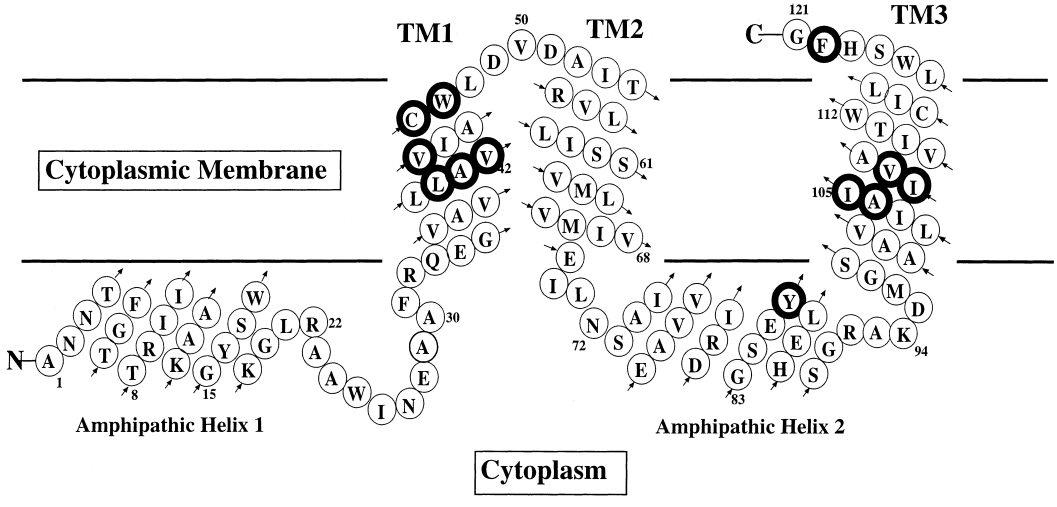

Fig. 1. Sequence and membrane topology of *E. coli* diacylglycerol kinase. DAGK is known to be a largely helical membrane protein [19] which spans the membrane three times with the sequential locations of its three transmembrane segments being approximately as shown [8]. DAGK functions as a homotrimer [7], the center of which is believed to be a bundle of three TM2 segments [9]. The residues for which cysteine substitution mutants were examined in this work are shown in bold. It should be noted that the two native cysteines in DAGK were mutated to alanine in all of the mutants examined in this work and that the 'Cys-less' DAGK mutant is fully active.

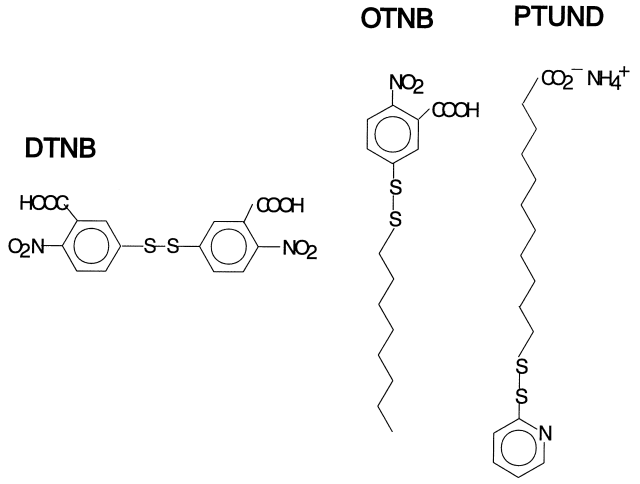

Fig. 2. Chemical structures of the activated disulfide reagents used in this work.

there will be strong steric and entropic forces which can be predicted to confer a modest preference for the interior of bilayers to the dithiopyridine moiety. Certainly, it is safe to say that the *probability* that the reactive dithiopyridine moiety of PTUND will be found in the membrane interior at any given moment is much higher than for OTNB or DTNB.

Experimental support for the above predictions is provided by two lines of evidence. First, as expected, the model reaction of free molecular cysteine with PTUND in mixed micelles was observed to be much slower than with OTNB which, in turn, was a little slower than with DTNB (Table 1). Secondly, the polarities of the critical dithiopyridine and dithionitrobenzoate moieties were quantitated by measuring solvent partitioning coefficients for DTNB and 2,2′-dithio-bis-pyridine (Table 2). Water/heptane partitioning data can be interpreted to reflect the relative preferences of these disulfides for the membrane interior relative to the aqueous phase [18]. Water–octanol partitioning can be interpreted to reflect preferences for the membrane interface relative to the bulk aqueous phase. As shown by the data of Table 2, DTNB has a relatively low tendency to partition from water into either a protic (octanol) or aprotic (heptane) organic solvent. On the other hand, dithio-bis-pyridine shows a strong preference for both organic solvents relative to water, with a modest preference (as predicted above) for octanol relative to heptane.

3.2. Modification of single-cysteine sites of diacylglycerol kinase

Samples containing identical concentrations of each purified DAGK mutant in DM/POPC mixed micelles were prepared as described in Section 2. DAGK mutants were functional under the specific conditions used. The advantage of running reactions in mixed micelles rather than in vesicles is that mixed micelles are optically transparent, such that the evolution of absorbance at 343 or 412 nm during the reactions can be directly monitored. 1 ml samples of each mutant at 0.07 mM were successively reacted with 0.33 mM concentrations of DTNB, OTNB, and PTUND. Examples of reaction time traces are presented in Fig. 3.

Initial rates were measured from the reaction time courses for each mutant/reagent combination. While no effort was made to establish reaction order or to measure true rate constants, for a given reagent it is possible to meaningfully compare initial rates for different cysteine sites because reactions were run under identical conditions from mutant to mutant. Rates are given in Table 1. All three reagents react most rapidly with residues believed to be located in the aqueous phase above the micellar interface (sites 120 and 86, see Fig. 1), with DTNB exhibiting much faster rates than OTNB and PTUND. Residues located in the membrane near the interface (46 and 47) reacted most rapidly with DTNB even though it is the most polar reagent, followed by intermediate reactivity with OTNB and, finally, very slow reactivity with PTUND. Most remarkably, membrane-buried sites (40–43, 105–107) reacted much more rapidly with OTNB than the more apolar PTUND. Indeed, for buried sites, DTNB reactions were generally more rapid than for PTUND. Membrane-buried sites in transmembrane segment 1 were generally much more reactive than were membrane-buried sites in transmembrane segment 3. All of these observations were bourne out when reactions were repeated at 4°C of all three disulfide reagents with single-cysteine mutants 40, 106, 107, and 120 (data not shown). It also should be noted that the lower reactivity of PTUND

Table 1
Initial reaction rates (micromolar/s) for modification of 0.07 mM DAGK or small molecule thiols by activated disulfide reagents (0.33 mM) at 30°C and pH 6.5 in DM/POPC mixed micelles

DAGK cysteine position or thiol compound	Initial rates as function of disulfide reagent		
	DTNB	OTNB	PTUND
Cysteine	>3	2 ± 1	0.014 ± 0.02
1-Octanethiol	0.20 ± 0.05	0.05 ± 0.01	0.001 ± 0.0008
40	0.05 ± 0.02	0.042 ± 0.013	0.015 ± 0.002
41	0.065 ± 0.01	0.014 ± 0.002	0.008 ± 0.002
42	0.06 ± 0.02	0.038 ± 0.008	0.008 ± 0.002
43	0.07 ± 0.02	0.038 ± 0.006	0.010 ± 0.002
46	0.031 ± 0.003	0.015 ± 0.002	0.002 ± 0.001
47	0.45 ± 0.06	0.024 ± 0.004	0.007 ± 0.002
86	>3	0.063 ± 0.013	0.02 ± 0.01
104	0.002 ± 0.001	0.005 ± 0.001	<0.001
105	0.004 ± 0.002	0.015 ± 0.002	0.004 ± 0.001
106	0.002 ± 0.001	0.004 ± 0.004	0.001 ± 0.001
107	0.002 ± 0.001	0.003 ± 0.001	<0.001
120	>2	0.4 ± 0.2	0.045 ± 0.010

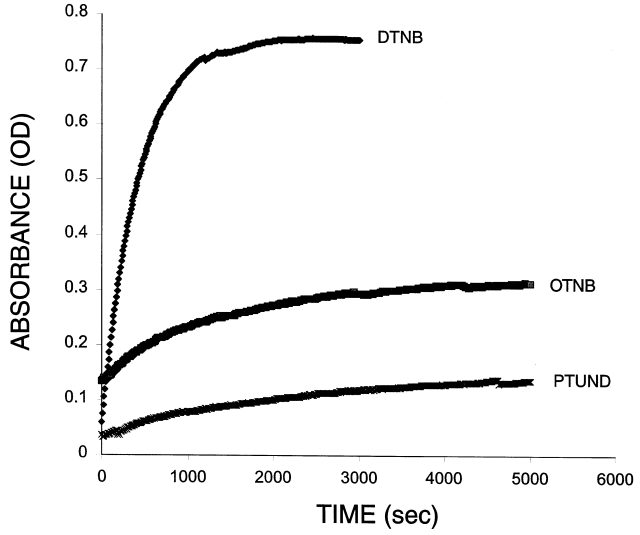

Fig. 3. Time traces of the reactions of W47C DAGK with all three disulfide reagents. Reactions were carried out in mixed micelles as described in Section 2. These traces were corrected to remove the effects of spontaneous reagent hydrolysis.

relative to OTNB or DTNB is not due to intrinsic differences between the thiopyridine moiety as a leaving group relative to thionitrobenzoate, as confirmed by model reactions of small molecule thiols with DTNB and 2,2′-dithio-bis-pyridine in solvents of varying polarity (data not shown).

The very slow rates observed between PTUND and membrane-buried sites on DAGK led us to test what the rate of reaction between an activated disulfide reagent and a small molecule thiol would be in an anhydrous solvent. 0.1 mM 11-thio-undecanoic acid was reacted with 0.6 mM 2,2′-dithio-bis-pyridine in heptane at 30°C (concentrations slightly higher than the DAGK and disulfide reagent concentrations used in this work). The initial relative rates for this reaction in the absence or presence of 2 mM of the base catalyst, dimethylaminopyridine, were 0.004 and 0.005 micromoles/s, respectively. Because of similarities in reaction conditions and reactant concentrations, these relative rates can be compared to the rates of Table 1. These rates are quite low and are similar to the rates of reaction of PTUND with some of the buried membrane sites (e.g. 105 and 106). Very slow reactions were also observed between all three disulfide reagents and octanethiol in both heptane and octanol in the presence of a base (data not shown). The explanation for why these thiol–disulfide exchange reactions were so slow in organic solvents is the mechanistic need for the sulfhydryl moiety to lose a proton to form a thiolate which can attack the activated disulfide bond. In a low dielectric medium such as a membrane interior there is a large energy barrier to anion formation.

4. Discussion

In this study we examined a total of 12 sites on DAGK, eight of which are believed to be fairly deeply embedded in the membrane and which include both lipid and protein-facing positions (see Fig. 1 and Section 1). Based on the number of sites examined, it seems possible to make some generalizations regarding the results. Contrary to expectation, it was observed that matching modification reagent hydrophobicity to the expected membrane location of protein cysteines did not result in enhanced rates relative to reactions with sites less well matched. Instead, the most highly water-exposed sites reacted most rapidly with all reagents regardless of reagent hydrophobicity. For example, the most hydrophobic reagent (PTUND) reacted much more rapidly with surface-exposed sites (86 and 120) than it did with any sites in the membrane. Moreover, deeply buried sites generally reacted most slowly with PTUND. Indeed, for membrane-buried sites 40–43, the most polar reagent (DTNB) reacted most rapidly. Taken together, these results indicate that there is little correlation between reactivity and reagent hydrophobicity for cysteine sites located in the membrane.

The above conclusion is surprising. If thiol exchange takes place deeply within the membrane-mimetic phase for buried cysteines, then the most rapid reactions would be expected for those reagents having the highest probability of being located in the membrane interior. Since this was clearly not observed for sites 40–43, this leads to the disturbing notion that some membrane-buried sites may *not* react primarily within the interior of the membrane-mimetic phase, particularly when reacting with polar reagents. Instead, such sites may react with polar reagents predominantly when normally buried cysteine sites make motional excursions to hydrated regions of the interface. For sites 40–43 this would explain the low reactivity of the apolar PTUND and the relatively high reactivity of the other reagents. That disulfide reactions occur only very slowly in the interior of the membrane is supported by the low rates observed for model reactions run in organic solvents (see Section 3). Moreover, because the reactive moiety of PTUND is normally membrane buried, there is only a low probability that it would chance to meet a normally buried cysteine in the hydrated interfacial region, whereas the more polar reagents maintain much higher local concentrations in that region.

The slow but sometimes observable reactions of PTUND with positions 104–107 may reflect the actual membrane interior reaction rates. This is supported by the fact that the observed rates were similar to or even slower than the observed rates for model chemical reactions in heptane (see Section 3). If so, then the especially low PTUND reactivity of sites 104 and 107 may suggest that these sites are disposed toward a protein interface rather than facing the lipid phase, while the converse would hold for sites 105 and 106. This is feasible given what is presently believed about the structure of

Table 2
Water–solvent partitioning coefficients for DTNB and 2,2′-dithio-bis-pyridine at 25°C and pH 6.5

Solvent pair	$K_{partition}$, 5,5′-dithio-bis-2-nitrobenzoate	$K_{partition}$, 2,2′-dithio-bis-pyridine
Water–heptane	94 ± 9	0.054 ± 0.005
Water–octanol	26 ± 3	0.026 ± 0.003

$K_{partition}$ = (concentration in buffered water)/(concentration in solvent) at equilibrium.

DAGK (see Section 1). We emphasize, however, that these results are merely suggestive.

The results indicated that buried sites (40–43) on transmembrane segment 1 were much reactive with all reagents tested than positions 104–107 on the third transmembrane segment. This may indicate that the first transmembrane segment has greater mobility than the third and thus undergoes more frequent liaisons with polar reagents in hydrated regions of the interface. This notion may find some support from previous studies showing that DAGK's functionality was maintained even when the first transmembrane segment (but not the third) was mutated to polyalanine [10].

In conclusion, the results of this paper strongly suggest that for DAGK the rates of reaction of membrane-buried cysteine sites with disulfide reagents are extremely slow in the interior of the membrane, so slow that the rates at which these sites react upon undergoing motional excursions to the interface may sometimes become a significant contributor to the overall reaction rate. Whether this indicates that DAGK is an unusually dynamic membrane protein or whether this observation may be relevant to many other membrane proteins remains to be determined. Certainly, in the case of DAGK's first transmembrane segment our results suggest that it would be very difficult to use chemical modification rates to distinguish with certainty between membrane-buried sites which are facing lipid versus membrane-buried sites which are disposed toward the protein interior.

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