Active Sites of Diacylglycerol Kinase from *Escherichia coli* Are Shared between Subunits†

Francis W. Lau, Xing Chen, and James U. Bowie*

Department of Chemistry and Biochemistry and Laboratory of Structural Biology and Molecular Medicine, UCLA, 405 Hilgard Avenue, Los Angeles, California 90095-1570

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ABSTRACT: We show that residues from different subunits participate in forming the active site of the trimeric membrane protein diacylglycerol kinase (DGK) from *Escherichia coli*. Five likely active-site mutants were identified: A14Q, N72S, E76L, K94L, and D95N. All five of these mutants possessed significantly impaired catalytic function, without evidence of gross structural alterations as judged by their similar near-UV and far-UV circular dichroism spectra. We found that mixtures of either A14Q or E76L with N72S or K94L possessed much greater activity than the mutant proteins by themselves, suggesting that Ala14 and Glu76 may be on one half-site while Asn72 and Lys94 are on another half-site. Consistent with the shared site model, we also found that (1) peak activity of A14Q and N72S subunit mixtures occur at equimolar concentrations; (2) the maximum activity of the A14Q and N72S mixture was 20% of the wild-type enzyme, in good agreement with the theoretical maximum of 25%; (3) the activity of mutant subunits could not be recovered by mixing with the wild-type subunits; (4) a double mutant, A14Q/N72S, bearing mutations in both putative half-sites was found to inactivate wild-type subunits; (5) the concentration dependence of inactivation by the A14Q/N72S mutant could be well described by a shared site model for a trimeric protein. Unexpectedly, we found that the single mutant D95N behaved in a manner similar to the double mutant, A14Q/N72S, inactivating wild-type subunits. We propose that Asp95 plays a role in more than one active site.

The integral membrane protein, diacylglycerol kinase (DGK)[1] from *Escherichia coli*, catalyzes the conversion of diacylglycerol and MgATP to phosphatidic acid and MgADP. Figure 1 shows the secondary structure and topology proposed by Smith et al. (*1*), which is consistent with sequence analysis, β-lactamase/β-galactosidase fusion experiments, CD spectroscopy, and FTIR spectroscopy (*2*−*4*). It has been shown that DGK is trimeric with three complete active sites and consists of subunits that are each 121 residues in length, after posttranslational processing of the N-terminal (*3*, *5*−*8*).

The finding that DGK is a trimer raises the question of whether the active sites are contained within each monomer or whether they are composed of residues from different subunits. To distinguish between these two possibilities, we have adopted a subunit mixing approach pioneered by Schachman and colleagues (*9*, *10*). In this method, inactive enzyme variants are prepared by engineering mutations in each half-site. Mutants with side-chain substitutions located on the same half-site will not complement when mixed. However, mixing mutants with substitutions on different halves of an active site can partially restore activity since a subset of the hybrid molecules that form will contain wild-type active sites.

In many cases, studies on shared active sites have benefited from the existence of high-resolution crystal structures, making the selection of which residues to alter straightforward. In the case of DGK, however, the three-dimensional structure is not known. To select likely active-site residues, we made use of our previous mutational analysis where we examined the tolerance of every residue in the sequence to amino acid substitutions (*11*). In the earlier work, the gene was mutagenized and sequences that encoded active enzymes were identified. This approach created a large library of 614 neutral amino acid substitutions. While the vast majority of residues in DGK tolerated a variety of different side chains, eight positions were identified which tolerated no changes. The positions of the invariant residues are shown on Figure 1. Because it seemed likely that some of the invariant residues were located in the active site, we introduced mutations at each of these positions. Subunit-mixing experiments performed on a subset of these mutant proteins suggest that the active site of DGK is indeed composed of residues from different subunits.

MATERIALS AND METHODS

Generation of Mutants. Mutations were introduced into a synthetic DGK gene on plasmid pSD004 (*4*). This plasmid contains restriction sites throughout the gene sequence.

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* To whom correspondence should be addressed. Phone: (310) 206-4747. Fax: (310) 206-4749. E-mail: bowie@ewald.mbi.ucla.edu.

[1] Abbreviations: DGK, diacylglycerol kinase; DM, *n*-decyl-β-D-maltoside; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; far-UV CD, far-ultraviolet circular dichroism; near-UV CD, near-ultraviolet circular dichroism; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TM1, transmembrane segment 1; TM2, transmembrane segment 2; TM3, transmembrane segment 3.

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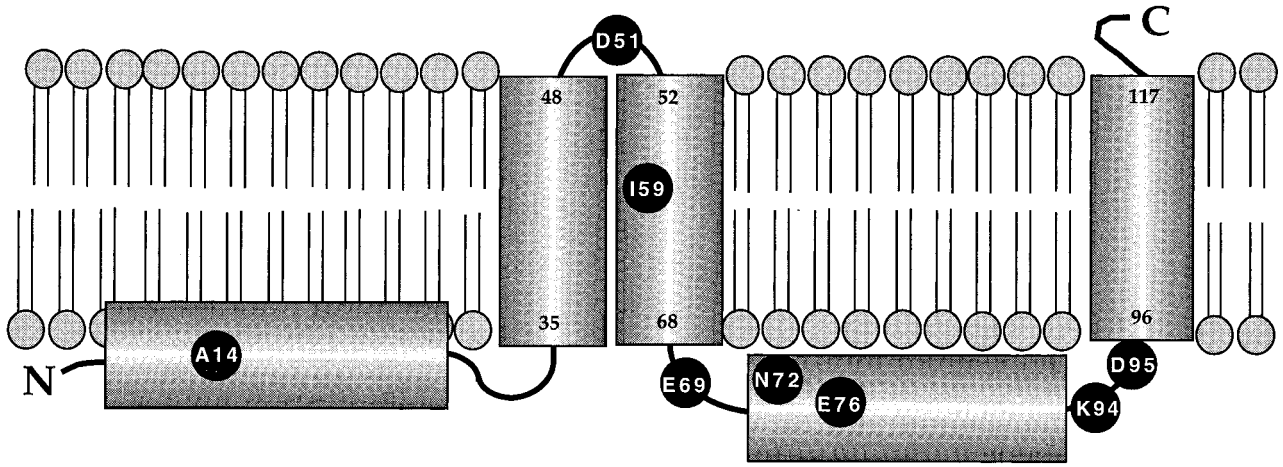

FIGURE 1: Topological model of DGK with invariant residues. The model is from Smith et al. (*1*). The putative α-helices of the protein are depicted as rods. DGK is thought to consist of three transmembrane helices located within at residues 35−48, 52−68, and 96−117. The two cytoplasmic domains of the protein are also believed to be mostly helical and are located at residues 1−34 and 69−95. Black circles indicate the approximate positions of invariant residues.

Double-stranded oligonucleotide cassettes were synthesized in which each position of the targeted codon was replaced with an equal mixture of all four nucleotides. The oligonucleotide cassettes were then introduced into the synthetic gene by standard procedures (*12*). DNA sequencing of the entire cassette region using the Thermo Sequenase cycle sequencing kit supplied by Amersham was performed in order to identify mutations.

Preparation of Proteins. Proteins were purified as described previously (*4*) except that small quantities of aggregated protein were removed by separation on a Sephacryl S-100 column (Pharmacia) equilibrated with 10 mM PIPES (pH 7.0), 0.30 M NaCl, and 0.5% *n*-decyl-*β*-D-maltoside (DM). To obtain unfolded protein for the mixing experiments, protein samples were applied to a Sephacryl S-100 column equilibrated with 10 mM PIPES (pH 7.0) containing 1.0% SDS. In both cases, samples were stored at −80 °C until used.

Mixing and Refolding of DGK Proteins. Proteins at 57 μg/mL in 10 mM PIPES (pH 7.0), 1.5 mM DTT, and 1.0% SDS were mixed prior to initiating refolding. Refolding was accomplished by a 10-fold dilution of the mixtures into buffer containing 50 mM PIPES (pH 7.0), 50 mM DM, 2 mM bovine heart cardiolipin (Avanti Polar Lipids), and 1.5 mM DTT. Under these conditions, maximal recovery of activity (70−80% of expected) was achieved within 5 h.

Activity Assays. Kinetic parameters were obtained using a radioactive assay in the detergent Triton X-100 as described previously (*13*). Other enzyme assays were performed using either a colorimetric assay described previously (*4*) or a modified version adapted for use with a microtiter plate reader. In the modified version, reactions were performed in the individual wells of a microtiter plate instead of optical cuvettes; the reaction volume was reduced to 0.20 mL, and the reaction was initiated by addition of 10 μL of enzyme at approximately 5.7 μg/mL. Typically, multiple reactions were monitored simultaneously using a Molecular Devices Spectramax 340 PC microtiter plate reader and data processed using Softmax PRO software from Molecular Devices.

CD Spectroscopy. CD spectroscopy was performed using an AVIV 62DS spectropolarimeter. Protein concentrations of 0.57 mg/mL were typically used for both far-UV and near-UV CD spectra. In the case of far-UV CD spectra, a quartz cell with a 0.1 mm path length was used. The buffer consisted of 10 mM PIPES (pH 7.0) and 1.0% DM. For each spectrum, 20 scans were acquired at 1.0 nm intervals using a 1.0 nm bandwidth and 1 s time constant. Near-UV CD spectra were taken using similar conditions except that a 10 mm quartz cuvette was used and the buffer contained 10 mM PIPES (pH 7.0), 0.3 M NaCl, and 0.5% DM.

RESULTS

Generation of Potential Active-Site Mutants. To test for shared sites using the subunit mixing approach, it was first necessary to generate mutations at positions likely to be located within the active sites. In our earlier neutral mutagenesis experiments, we found eight sites that were intolerant to substitution and were therefore likely to be playing important roles in protein structure and/or function (*11*). These residues were targeted for mutagenesis (see Figure 1).

Mutagenesis was facilitated by a previously constructed synthetic gene that possessed unique restriction sites throughout the coding sequence (*4*). Each position was mutated by preparing an oligonucleotide cassette in which all three nucleotides in the targeted codon were replaced by an equal mixture of all four nucleotides. After introduction of the mutagenic cassette into the synthetic gene, mutations in individual clones were identified by sequencing the entire cassette region.

The activity of each mutant enzyme was rapidly assessed using an assay that can be performed on individual colonies (*11*). The activities of the mutants in the crude colony assay are listed in Table 1. As expected, most substitutions at these eight residues are strongly deleterious. An exception is position 59. Although apparently invariant in our earlier neutral mutagenesis experiments, position 59 was in fact quite tolerant to sequence substitutions. We suspect that, in our earlier work, mutations were suppressed at this position for reasons that have nothing to do with enzyme activity. Perhaps the position was not mutagenized as effectively as we thought. Clearly, position 59 is not critical for activity as previously suspected. At position 51, we found that most

Table 1: Activity of Mutant Enzymes

position	wild-type residue	activity in colony assay		
		100−30%	20−5%	<5%
14	A	S,C,V	Y,P,**Q**	L,E
51	D	P	T,M,E,C	K,R,V,S
59	I	A,S,W,L,F,T,V	G	K
69	E		**C**,S,A	L,R,F,P,V,G
72	N			W,L,I,F,V,T,C,**S**,A
76	E			W,Y,R,V,G,**L**,H,S,P,K,M
94	K			**L**,I,R,H,P,V,D,A,G
95	D		E,Q,S,T,I,L	R,Y,P,**N**,G

[a] Mutant enzyme activities were measured using a colony assay previously described (*11*). Bold letters denote mutants that were purified for further study. Values are given as percent of wild-type activity.

Table 2: Kinetic Parameters of Mutants Enzymes

protein	K_m, MgATP(mM)	K_m, DAG (mol %)	V_{max} (μmol min^{-1} mg^{-1})
wt	0.12 ± 0.01	1.2 ± 0.2	48 ± 8
A14Q	0.91 ± 0.34	2.2 ± 0.3	4.1 ± 1.4
E69C	0.33 ± 0.18	2.0 ± 0.5	0.22 ± 0.10
N72S	0.44 ± 0.04	2.2 ± 0.3	0.39 ± 0.18
E76L	0.14 ± 0.01	5.0 ± 1.3	0.03 ± 0.02
K94V	1.5 ± 0.2	4.9 ± 1.1	1.12 ± 0.48
D95N	2.1 ± 0.4	5.6 ± 1.9	1.11 ± 0.70

mutations were deleterious, but D51P was completely active. In fact, the D51P variant was purified and found to possess a specific activity 20% higher than the wild-type protein (not shown). Asp51 is predicted to be in a turn between TM1 and TM2, and it is conceivable that Asp51 is critical for making this turn (*1*). The fact that both Asp and Pro can function perfectly at this position suggests that position 51 is not an active-site residue and is instead playing a structural role. For the purposes of this study, we chose to focus on the residues likely to be important for catalysis and purified the following set of six mutant proteins: A14Q, E69C, N72S, E76L, K94L, and D95N.

Kinetic Parameters of DGK Mutants. As shown in Table 2, all of the purified mutant proteins have substantially altered steady-state kinetic parameters as would be expected for residues in the active site of the enzyme. All the mutants reduce V_{max} by at least 1 order of magnitude, indicating a role for each of these side chains in transition-state stabilization. For mutants A14Q, E69C, N72S, and E76L, the effects of side-chain modification appear to be specific for transition-state stabilization, rather than substrate binding, since the effects on K_m are relatively modest. The mutants K94L and D95N, however, are apparently important for binding the MgATP substrate as well as the transition state since the K_m values for MgATP are raised 13- and 18-fold, respectively.

Structural Integrity. Because kinetic parameters can be altered by global conformational changes, we looked for evidence of structural changes in the mutant enzymes. To detect any changes in secondary structure, far-UV CD spectra were taken of all the mutant proteins along with wild-type DGK. As shown in Figure 2, no significant differences in the far-UV CD spectra are evident, indicating that the gross secondary structure of the protein is unaltered by the mutations. To investigate alterations in tertiary structure, near-UV CD spectra were recorded. As shown in Figure 3, only A14Q and E69C show significant deviations from the wild-type spectrum. In the case of A14Q, the shape of the

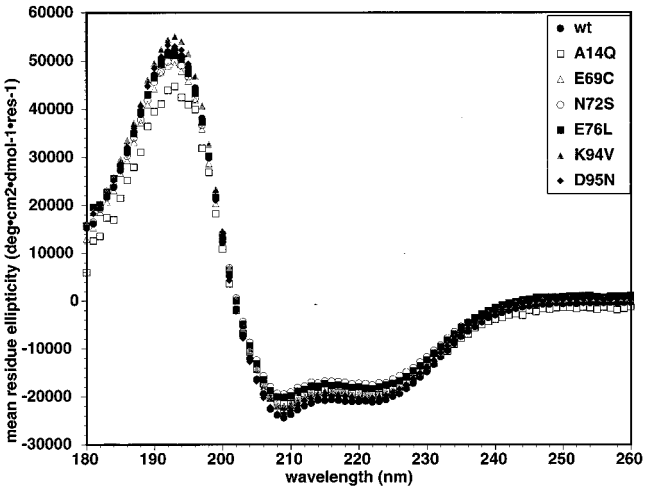

FIGURE 2: Far-UV CD spectra. Protein concentrations of 0.57 mg/mL in 10 mM PIPES (pH 7.0) containing 1.0% DM were used. Samples were measured using a quartz cuvette with a path length of 0.10 mm. Each spectrum represents the average of 20 scans taken at 1.0 nm intervals using a 1.0 nm bandwidth with a 1 s time constant.

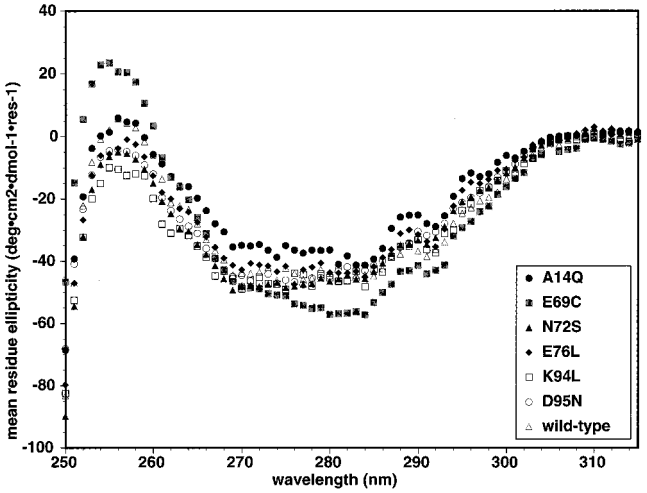

FIGURE 3: Near-UV CD spectra. Protein concentrations of 0.57 mg/mL in 0.30 M NaCl, 10 mM PIPES (pH 7.0) containing 1.0% DM were used. Samples were measured using a quartz cuvette with a path length of 10 mm. Each spectrum represents the average of 20 scans taken at 1.0 nm intervals using a 1.0 nm bandwidth with a time constant of 1 s.

spectrum is quite similar to the wild-type enzyme, but slightly reduced in intensity. The purified A14Q protein was found to contain small amounts of aggregated protein that was similar in size to the native protein and therefore could not be completely removed by the gel-filtration chromatography step. Thus, we believe that the reduced intensity of the A14Q spectrum is not due to structural alterations, but instead to the presence of unfolded aggregate in the sample. The shape of the E69C spectrum, however, is markedly different, particularly the positive peak at 255 nm. We also found that E69C migrated anomolously in SDS−PAGE (not shown), indicating that the mutant has an altered structure even in SDS. Although these changes could be the result of local structural alterations, we cannot rule out the presence of a more global conformational change caused by this mutation.

Complementation of Mutants. According to a shared site model, subunits bearing deleterious mutations on the opposite

a.

Shared-site Model

Experiment	Before Mixing a^3	Before Mixing b^3	After Mixing a^3	After Mixing a^2b	After Mixing ab^2	After Mixing b^3	Fractional Activity
I	(0)	(0)	(0)	(1/3)	(1/3)	(0)	a(1-a)
II	(1)	(0)	(1)	(2/3)	(1/3)	(0)	a
III	(1)	(0)	(1)	(1/3)	(0)	(0)	a^2

b.

Conformational Change Model

Experiment	Before Mixing a^3	Before Mixing b^3	After Mixing a^3	After Mixing a^2b	After Mixing ab^2	After Mixing b^3	Fractional Activity
I	(0)	(0)	(0)	(1/3)	(1/3)	(0)	a(1-a)
II	(1)	(0)	(1)	(1)	(2/3)	(0)	a(2-a)

FIGURE 4: Description of subunit mixing experiments. Depictions of both (a) a shared site model and (b) a conformational change model are presented. The different mixing experiments performed in this study are labeled in the first column with Roman numerals. The active site of DGK is shown as being composed of both a square and triangle. Filled triangles and squares represent defective halves of an active site. Subunits originating from different proteins are shown as either white or gray circles. Equations describing the expected fractional activity are also shown. Given the fact that DGK is a trimer (*3*), the distribution of the various species, which result from the mixing of two different proteins, is described by a cubic equation of the following form: $a^3 + 3a^2b + 3ab^2 + b^3 = 1$, where the parameters a and b represent the fraction of each subunit in the mixture such that $a + b = 1$. The expected fraction of the homotrimers are a^3 and b^3 while $3a^2b$ and $3ab^2$ denote the fractions of two possible hybrids which form. The coefficients represent the relative ratio of each species in the overall population (there are three ways to make an a^2b hybrid). The expected fractional activity of each species relative to wild-type DGK is shown in the figure by the number in parentheses below each species. For example, wild-type DGK containing three complete active sites has a fractional activity of 1, while a hybrid molecule with two complete active sites has a fractional activity that is two-thirds of the wild-type protein. The expected fractional activity for the entire mixture depends on both the activity of each species and the relative amounts in the mixture.Thus, for experiment I, the fractional activity is $(1)(0)(a^3) + (1/3)(3)(a^2b) + (1/3)(3)(ab^2) + (1)(0)(b^3)$, resulting in a fractional activity of $a(1 - a)$ when b is substituted by $1 - a$. In the case of the conformational change model (4b), circles represent properly folded subunits. Hexagons represent malformed subunits containing defective active sites, shown in black. In experiment I of panel b, a misfolded trimer, shown in gray, is mixed with a correctly folded one that contains a defective active site. A rescued subunit containing a functional active site is shown with a gray circle. Although it is theoretically possible for a properly folded subunit to restore activity to more than one active site, the mixing data shown in Figure 6 is fit by a model in which one properly folded subunit restores activity to only one misfolded subunit.

halves of a shared active site should be able to complement one another when mixed (see Figure 4a, experiment I). Those on the same half-site, however, will not complement. Having identified potential active-site residues, we next tested for restoration of activity upon subunit mixing. In our previous work, we showed that activity could be recovered from monomeric DGK in SDS after dilution into buffer containing the nondenaturing detergent DM (*4*). In this present study, mutant proteins were first denatured in SDS before they were mixed and renatured by dilution into buffer containing the nondenaturing detergent DM.

The results of the pairwise mixing experiment are displayed in Figure 5. The degree of complementation is given as the ratio of the activity of the mixture over the total expected activity from each protein which makes up the pair. A value larger than 1 indicates that enhancement of activity occurs upon mixing. As shown in Figure 5, A14Q complements E69C, N72S, K94L, and D95N but not E76L. The mutant E76L also complements E69C, N72S, and K94L, albeit less effectively than A14Q. When viewed through the shared site model shown in Figure 4, the results suggest that Ala14 and Glu76 could be located on one-half of a shared

Proteins	A14Q	E69C	N72S	E76L	K94L	D95N
A14Q						
E69C	8.8±2.1					
N72S	12.7±2.9	1.3±1.0				
E76L	0.9±0.1	2.9±0.3	3.1±0.3			
K94L	5.1±1.3	0.8±0.3	0.8±0.3	2.5±0.3		
D95N	4.1±1.2	1.1±0.7	1.7±1.1	1.3±0.1	0.4±0.2	

FIGURE 5: Complementation table. Equal quantities of each protein in SDS were mixed and refolded as described in the Materials and Methods. The degree of complementation is given as the ratio of the final activity over the initial activities from the contributing proteins. Values of 1 indicate no complementation and are shown with white boxes. Values between 2 and 4 are shown in light gray and values 5 and greater are shown in dark gray.

site and Glu69, Asn72, and Lys94 on the other half. Variability in the recovery of activity could be a result of differential refolding yields, incomplete mixing or mutations having partial affects on more than one site. D95N behaves anomalously, complementing A14Q but not E69C, N72S, or E76L and inhibiting K94L. The unusual behavior of D95N will be discussed further below.

Maximum Complementation Occurs at Equal Amounts of A14Q and N72S. To better characterize the nature of the mutant complementation, we measured activity as the molar ratio of the mutant subunits was varied. Mutants A14Q and N72S were chosen for this work since this pair displayed the highest degree of complementation. Figure 6 shows activity as a function of the ratio of A14Q and N72S subunits. As predicted by the shared site model (Figure 4a, experiment I), activity was found to reach a maximum when equal amounts of both A14Q and N72S are mixed. Furthermore, the peak specific activity is approximately 20% that of wild-type DGK. According to the shared site model, the maximum specific activity in this experiment should be 25% (*10*). Given the fact that only 70−80% of the protein successfully refolds in these experiments, our results agree quite well with theory.

Alternative Conformational Change Model. Although the data shown in Figure 6 fit a shared site model, it is possible that a conformational change model could also explain these data under a particular set of circumstances. Specifically, if every subunit from one mutant restored full activity to a subunit from the other mutant, the results seen in Figure 6 would be obtained. We therefore constructed a mathematical model for conformational change that would be consistent with the data in Figure 6. The resulting model, shown in Figure 4b, serves as a comparison guide in further experiments.

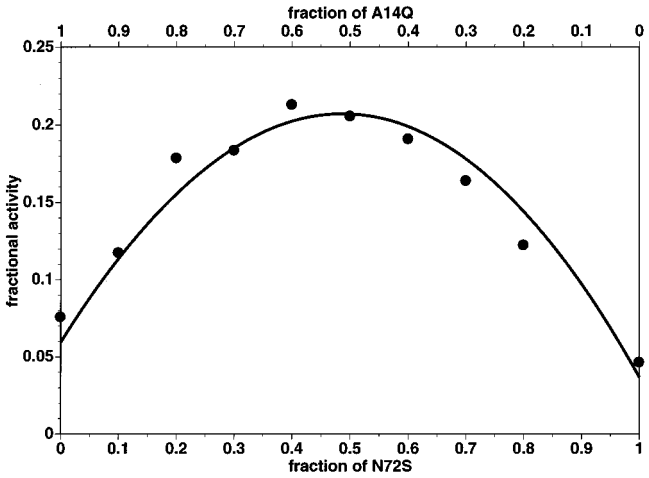

FIGURE 6: Complementation of A14Q by N72S. The fractional activity of mixtures containing differing amounts of A14Q and N72S are shown. Maximum activity occurs when equal concentrations of each mutant are mixed. Data were fitted to the fractional activity equation given in Figure 4, experiment I using maximal activity as the only adjustable parameter. Both the shared site model and the conformational change model are described by the same equation.

Table 3: Activities of Wild-Type and Mutant Subunit Mixtures[a]

proteins	specific activity (% of wild-type)
A14Q	49.5 ± 1.1
E69C	50.3 ± 3.0
N72S	51.3 ± 4.0
E76L	51.5 ± 2.6
K94L	54.8 ± 1.4
D95N	27.7 ± 1.2

[a] Equal amounts of wild-type DGK and each mutant were mixed and refolded as described in the Materials and Methods. the specific activities of each mixture are given in terms of percent of wild-type denatured and refolded in the same manner. Values given are the averages of three to four measurements. The uncertainty represents the range of values within each measurement.

Wild-Type DGK Is Unable To Rescue Mutants. According to the conformational change model, one subunit from a properly folded protein should completely rescue one misfolded subunit (Figure 4b, experiment II). Therefore, wild-type subunits should be able to rescue the mutant subunits. In mixtures of wild-type and mutant subunits, the specific activity of the mixture should then be higher than could be accounted for by the wild-type subunits alone. According to the shared site model, however, in mixtures of single mutant subunits with wild-type subunits, the activity should be proportional to the fraction of wild-type subunits (Figure 4a, experiment II). Thus, in equimolar mixtures of wild-type and mutant subunits, the shared site model predicts specific activity that is 50% of the wild-type activity and the conformational change model predicts 75%. As shown in Table 3, the specific activities are about 50% of the wild-type specific activity. The exception is the D95N/wild-type mixture, for which a suprisingly low specific activity was obtained. D95N appears to inactivate wild-type DGK. This result is discussed below.

To further illustrate the difference between the shared site and conformational change models, we examined the dependence of specific activity on the ratio of mutant to wild-type subunits in the mixture. In the conformational change model, activity would be higher than expected, given the

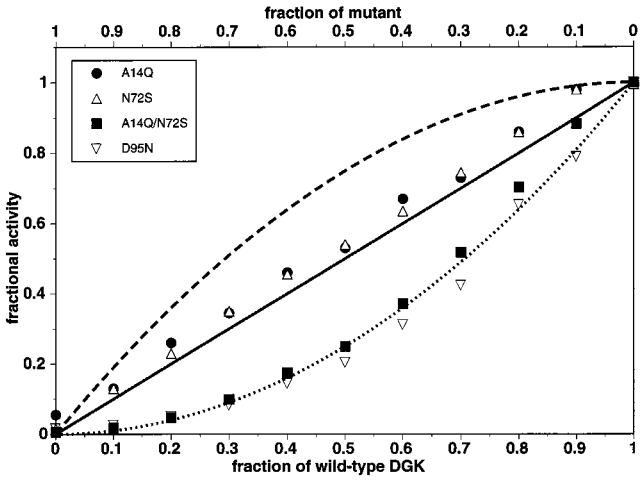

FIGURE 7: Mixing of A14Q, N72S, A14Q/N72S, and D95N with wild-type DGK. Increasing amounts of wild-type DGK were mixed with A14Q, N72S, A14Q/N72S, or D95N. The mixtures were refolded as described in the Materials and Methods and their activities measured. The dashed line represents results expected from the conformational change model. Results expected for the shared site model are shown using a solid line in the case of a single mutant and a dotted line in the case of a double mutant.

same fraction of wild-type subunits, since activity would be restored to the mutant subunits. Figure 7 shows activity as a function of the fraction of mutant subunits A14Q and N72S. Activity is linear with the fraction of wild-type subunits, consistent with the shared site model.

Mixing the Double Mutant A14Q/N72S with Wild-Type Abolishes Activity. In the shared site model, mixing wild-type protein with a double mutant bearing mutations on both halves of the shared active site would be expected to inactivate the wild-type subunits (Figure 4a, experiment III). Thus, in mixtures of wild-type subunits and double mutant subunits, the specific activity would be lower than expected based on the fraction of wild-type subunits. In the conformational change model, specific activity should generally be higher than expected (Figure 4b, experiment II). As seen in Figure 7, A14Q/N72S behaves like an ideal double mutant in the shared site model.

Asp95 Inactivates Wild-Type Subunits. Our initial mixing experiment with D95N and wild-type DGK (Table 3) showed that D95N inhibited activity. We characterized this behavior further by measuring the dependence of activity on different ratios of D95N to wild-type. The results, shown in Figure 7, show a striking resemblance to the concentration dependence expected for a double mutant bearing mutations in both half-sites (Figure 4a, experiment III). Similar behavior has been described previously and attributed to some sort of conformational change (*14*). However, no gross conformational changes were detected for D95N using CD spectroscopy. We believe that an alternative explanation exists to account for the behavior of D95N. A single mutation at Asp95 could effectively alter two active sites if Asp95 played a role in both sites (see below).

DISCUSSION

The results presented in this paper indicate that residues from different subunits make up each active site of DGK. In particular, Ala 14 and Glu76 are present on one-half site and Glu69, Asn72, and Lys94 are present on the other. First,

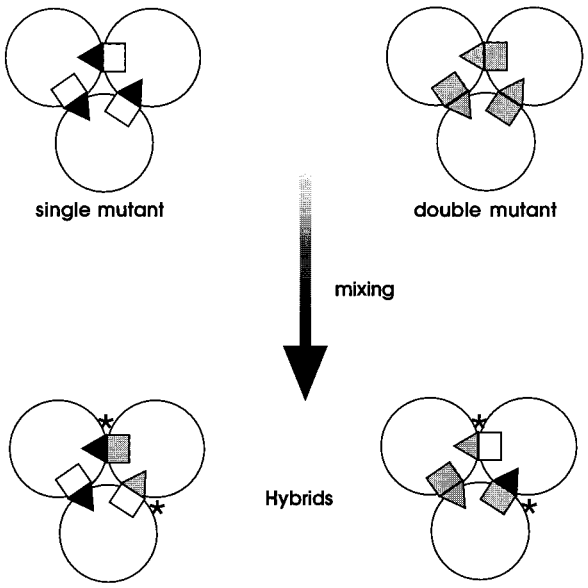

FIGURE 8: Double mutant and single mutant hybrids. The mixing of a double mutant with a single mutant is shown in terms of a shared site model. The hybrids which form as a result of mixing contain new active sites [indicated by an asterisk (*)] not seen in the original parent molecules. If Asp95 is present in more than one active site, it is like the double mutant in the figure.

with the possible exception of Glu69, these residues are likely to form part of the catalytic site since mutations at each of these positions result in significant reductions in catalytic efficiency without any apparent gross structural alterations. Second, when subunits bearing deleterious mutations at positions 14 or 76 are mixed with subunits bearing mutations at positions 72 or 94, activity is enhanced. This suggests that the mutated side chain on one subunit can be replaced by the wild-type side chain on another subunit. Third, mixing mutant subunits with wild-type subunits does not restore activity to the mutant subunit. This result suggests that the recovery of activity seen by mixing mutant subunits is not due to restoration of the active conformation. Fourth, mixing a subunit bearing mutations in both half sites (A14Q/N72S) with wild-type subunits inactivates the wild-type subunits. This behavior is also inconsistent with a mechanism that involves restoration of the active conformation in the mutant subunits. Moreover, the concentration dependence of inactivation is well described by the shared site model. Thus, the shared site model is completely consistent with the data presented.

The finding that mutations in another apparent active-site residue, Asp95, can inactivate wild-type subunits is particularly interesting. Although it is possible that the mutant D95N has an altered conformation that is transmitted to the wild-type subunit, no evidence for gross structural alterations was found in the near-UV CD spectrum. Moreover, the concentration dependence of wild-type subunit inactivation by D95N subunits is described very well by a model that assumes D95N is behaving like a double mutant and inactivating more than one-half-site. This result suggests that each Asp 95 may participate in more than one active site. Additional support for this hypothesis can be found in an apparent paradox: although D95N subunits inactivate wild-type subunits, we found that they were capable of restoring activity to A14Q subunits (see Figure 5). This result is consistent with the idea that D95N plays a dual role in each active site and, as

far as we know, cannot be explained by transmission of a deleterious conformational change. As shown in Figure 8, if subunits bearing mutations in both half-sites are mixed with single mutant subunits, hybrids are created containing two new types of active sites that are not present in either the double mutant or the single mutant. In one case, a new active site is formed with a wild-type half-site. If this new active site is more active than either the original single or the original double mutant, activity will be enhanced upon mixing. While we might have expected similar behavior with the other mixtures, there are many factors that could limit the relative enhancement of activity including the starting activities, the refolding yield, and which half-site bears the mutation. For example, if Asp95 plays a more important role in the Ala14 side of the active site compared to the Asn72 side of the active site, it will not enhance the activity of Asn72 as effectively. Thus, lack of activity enhancement in any particular mixture is not difficult to explain. It is the fact that we do see enhancement of activity with the A14Q and D95N mixture, combined with the clear inhibition of wild-type subunits by D95N that leads us to propose a dual role for Asp 95. To our knowledge, this has not been seen before and would require that the active sites of DGK be in close proximity to one another. Structural information will probably be required to verify this suggestion conclusively.

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