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NMR-Based Amide Hydrogen–Deuterium Exchange Measurements for Complex Membrane Proteins: Development and Critical Evaluation

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A method for measuring site-specific amide hydrogen–deuterium exchange rates for membrane proteins in bilayers is reported and evaluated. This method represents an adaptation and extension of the approach of Dempsey and co-workers (*Biophys. J.* 70, 1777–1788 (1996)) and is based on reconstituting ^{15}N-labeled membrane proteins into phospholipid bilayers, followed by lyophilization and rehydration with D_2O or H_2O (control). Following incubation for a time *t* under hydrated conditions, samples are again lyophilized and then solubilized in an organic solvent system, where ^{1}H–^{15}N HSQC spectra are recorded. Comparison of spectra from D_2O-exposed samples to spectra from control samples yields the extent of the H–D exchange which occurred in the bilayers during time *t*. Measurements are site specific if specific ^{15}N labeling is used. The first part of this paper deals with the search for a suitable solvent system in which to solubilize complex membrane proteins in an amide "exchange-trapped" form for NMR quantitation of amide peak intensities. The second portion of the paper documents application of the overall procedure to measuring site-specific amide exchange rates in diacylglycerol kinase, a representative integral membrane protein. Both the potential usefulness and the significant limitations of the new method are documented. © 2000 Academic Press

Key Words: **hydrogen–deuterium amide exchange; membrane proteins; site-directed mutagenesis; diacylglycerol kinase; NMR.**

INTRODUCTION

Because amide hydrogen exchange rates depend on protein structure, hydrogen–deuterium (H–D) and hydrogen–tritium (H–T) exchange measurements have long been recognized as a rich source of protein structural information (*1–5*). There are a variety of experimental methods used for quantitation of hydrogen exchange in solution and membrane proteins. These methods can be categorized as low-, medium-, and high-resolution techniques. Low-resolution methods are useful for determining the bulk hydrogen exchange rate for an entire protein. Both spectroscopic and radioisotopic methods exist for measuring bulk exchange rates (*5–9*). A medium-resolution method developed by Smith and Zhang (*10, 11*) employs the combination of mass spectrometry and proteolysis to quantify levels of bulk isotopic exchange within resolvable protein *segments.* Currently, only NMR spectroscopy yields high-resolution site-specific H–D exchange rates for proteins (*1, 12–16*).

Integral membrane proteins represent a class of proteins for which the capacity to make site-specific amide exchange (AE) measurements is very desirable. There are examples of small membrane proteins solubilized in detergent micelles or organic solvent mixtures where AE rates in micellar or solvent mixtures have been directly measured by NMR (*17–23*). Such studies have required total assignment of the amide proton resonances. For larger membrane proteins, such direct measurements will often not be feasible because of the large effective molecular weights of protein–detergent aggregates and because organic solvent mixtures unfold many membrane proteins. Both of these problems seriously hinder necessary total assignment of the amide proton resonances.

For membrane proteins inserted into lipid bilayers, *direct* measurements of site-specific AE rates are presently not possible because of the difficulties associated with obtaining high-resolution proton NMR spectra of large molecules firmly embedded in lipid bilayers. For this reason, Dempsey and co-workers (*15, 24*) have developed a clever "exchange-trapping" approach in which a bilayer-associated membrane protein is exposed to H–D exchange conditions, followed by trapping of the in-bilayer amide exchange state via transfer of the membrane protein out of the bilayer and into an effectively exchange-inert organic solvent system in which the percentage of remaining protons at each amide site can be assessed by solution NMR methods. This approach has been demonstrated to be successful for small membrane proteins, for which spectral assignments in organic solvents can readily be made (*15, 24*). However, in the case of larger membrane proteins it will often not be possible to assign all amide proton resonances when the protein is solubilized in an organic solvent mixture. For example, in the case of *E. coli* diacylglycerol kinase, organic solvent systems capable of solubilizing the protein at NMR concentrations also unfold the protein (*25*), inducing widespread NMR spectral degeneracy. In addition, the solvent systems employed by Dempsey and co-workers may not be effective at solubilizing all membrane proteins. This paper

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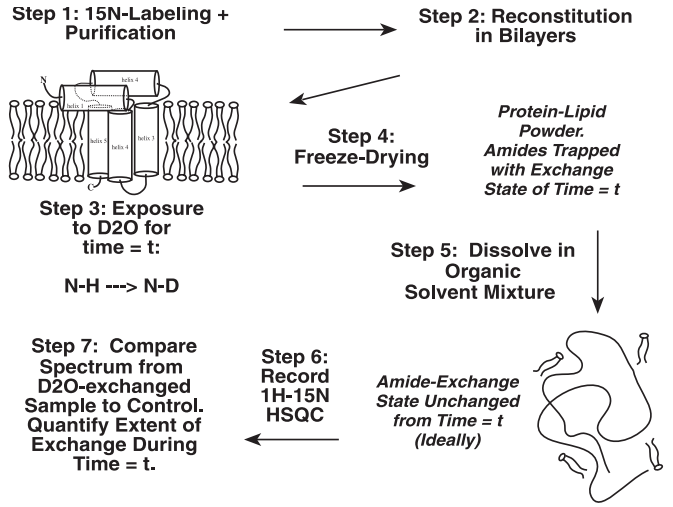

FIG. 1. Schematic of the amide exchange measurement procedure tested in this work.

documents the development of a procedure which builds upon the Dempsey exchange-trapping approach to permit site-specific in-bilayer H–D exchange rates to be measured for even larger membrane proteins.

RESULTS

General Strategy

Figure 1 outlines the experimental approach to measuring amide exchange rates which is the subject of this paper. This procedure draws heavily upon the work of Dempsey (*15, 24*) and, to a lesser degree, Roder and co-workers (*14*). Following ^{15}N labeling and membrane reconstitution of the protein, amide exchange is allowed to evolve under conditions best approximating the native environment: with the membrane protein in lipid bilayers. Amide exchange is not *directly* assessed while the protein is still in bilayers. Instead, the amide exchange state of the in-bilayer protein at time *t* is trapped by freeze-drying, followed by redissolution of the protein into an organic solvent system in which further amide exchange is suppressed and in which "solution NMR" conditions pertain. At this point, comparing HSQC spectra from exchanged and control samples permits quantitation of the exact degree of amide exchange occurring in the bilayer samples during time *t*. In the case of uniformly labeled membrane proteins, assignments of the HSQC spectra may not be feasible and the proposed method would be expected to yield, at best, the bulk exchange rate. However, if site-specific ^{15}N labeling is possible, then assignment of the HSQC spectrum is trivial and the proposed method will provide direct exchange measurements for the labeled site.

Diacylglycerol Kinase as a Test Case

The test system employed in this paper is *E. coli* diacylglycerol kinase (DAGK). DAGK functions in membranes as a noncovalent trimer of identical 13 kDa subunits (121 residues each (*25*)). DAGK is an almost exclusively helical protein in which each subunit contains three transmembrane helices (*26, 27*). DAGK has been subjected to an extensive preliminary examination by NMR in both micelles and organic solvent mixtures (*25, 28*). In micelles, DAGK folds properly but exhibits extremely broad NMR spectra. In organic solvent mixtures, DAGK is largely unfolded and gives spectra characteristic of a molten globular conformational state. In neither micelles nor organic solvent systems does the prospect of making total resonance assignments appear to be promising (*25*). Preliminary 800 MHz TROSY (*29*) measurements (Sanders, unpublished) have not dramatically changed this assessment. Because of its structural complexity, DAGK represents a rigorous test system.

Screening of Potential Organic Solvent Mixtures and Testing Solvent Systems

An appropriate solvent mixture in which to solubilize membrane proteins for the NMR step of the AE method would be a system which (1) fully solubilizes membrane proteins at >0.1 mM concentrations, (2) does not allow additional H–D exchange to take place, and (3) solubilizes the protein in a form such that reasonably sharp amide peaks are observed (although not necessarily well resolved).

We observed that organic solvent systems previously documented for use in solvent trapping AE studies (*14, 15, 24*) were not effective at solubilizing DAGK. This led us to search for a new system. We previously screened about 2000 solvent systems for their ability to solubilize DAGK or model membrane polypeptides (*25, 30*). Those previous results provided the basis for selection of the 74 solvent systems examined in the present study. Various combinations of solvents containing exchangeable protons (e.g., water, methanol, hexafluoro-2-propanol, and trifluoroethanol), *aprotic* polar solvents (e.g., dimethyl sulfoxide, dimethylformamide, and tetrahydrofuran), and *aprotic* nonpolar solvents (e.g., chloroform and benzene) were evaluated. Solvent mixtures were evaluated at basic (ammonium hydroxide), neutral, and acidic (acetic acid, formic acid, and trifluoroacetic acid) pH. A detailed list of systems tested is available upon request from the authors.

Only about 20 of the solvent systems examined were able to totally dissolve DAGK (from a detergent/DAGK powder) at a concentration of 0.2 mM. Some solvents (e.g., mixtures containing dimethyl sulfoxide) dissolved DAGK at higher temperature (50°C) but not at room temperature (25°C). Of the 20 mixtures which completely dissolved DAGK, only 5 gave high or satisfactory *S*/*N* spectra from DAGK (8 mg/ml concentration). These systems were (A) neat hexafluoro-2-propanol (HFIP), (B) benzene:HFIP 1:1 (vol:vol) + 1% formic acid (FA), (C) 2-propanol:H_2O 1:1 + 0.5% FA, (D) benzene:trifluoroethanol (TFE) 1:1 + 0.5% FA, and (E) TFE:H_2O 8:2 + 0.5% FA. Other mixtures yielded only very broad signals even

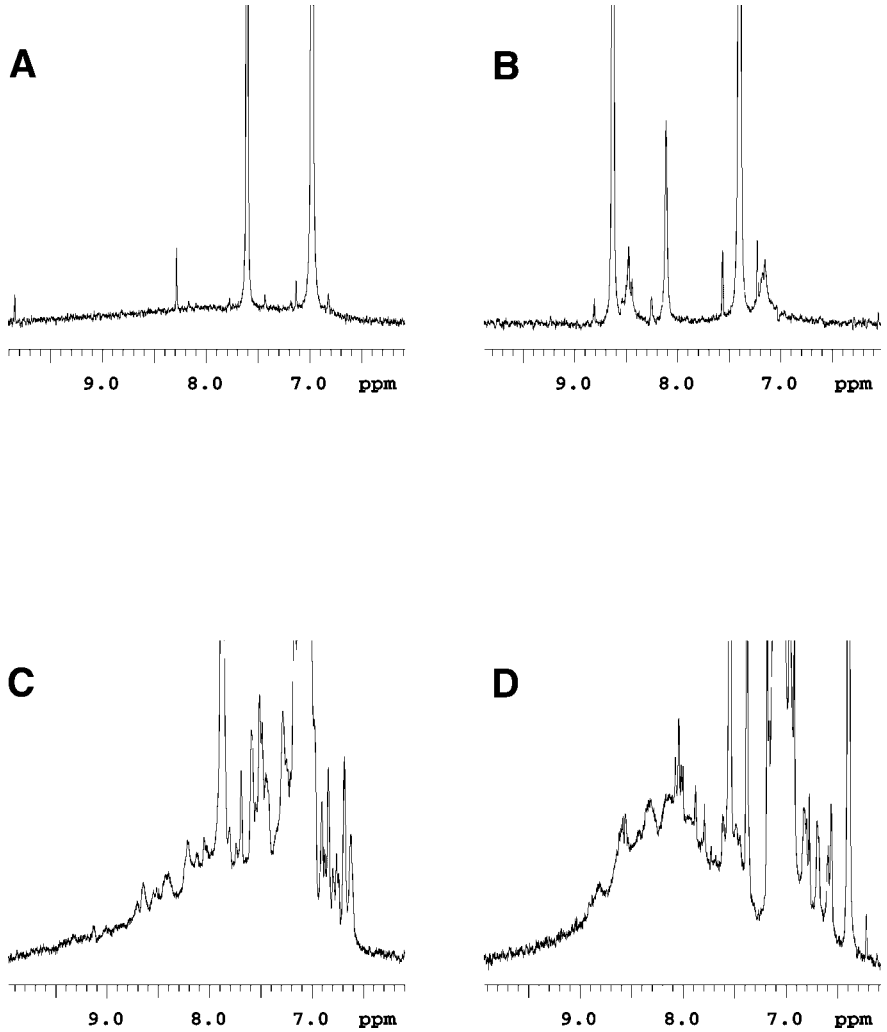

FIG. 2. 1-D ^{1}H NMR spectra (amide/aromatic region) of DAGK in representative solvent mixtures: (A) 100% dimethyl sulfoxide; (B) methanol:H_2O 6:4 + 0.5% formic acid; (C) 100% hexafluoro-2-propanol; (D) benzene:hexafluoro-2-propanol 1:1 + 1% formic acid. All spectra were acquired at 25°C.

when the temperature was elevated to 50°C. Figure 2 compares representative spectra from mixtures yielding reasonably high signal-to-noise to examples which did not. The only peaks observed in the latter cases are from small-molecule impurities or from the highly mobile poly-His tag of recombinant DAGK. It is significant that all of the solvent systems which were found to yield high signal-to-noise from DAGK contained at least 50% *protic* solvent. This is unfortunate since the presence of exchangeable solvent protons means that "post-trap" solvent–protein amide H–D exchange must remain a concern. The fact that four of five of the best systems also required the presence of formic acid to facilitate DAGK solubility is also a cause for concern since the low pK_a of formic acid dictates the effective pH will be below values at which unwanted solvent–amide H–D exchange is known to be minimized (*14*).

Because the five best solvent systems contain exchangeable protons, leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) was employed to evaluate the possibility of undesired amide H–D exchange in the five best solvent systems. Leucine enkephalin was chosen for these tests because it is unstructured, such that it should provide a stringent test of the degree of unwanted amide exchange because its amides will be fully exposed to solvent. Leucine enkephalin was dissolved in deuterated solvent mixtures and examined by 1-D ^{1}H NMR at various times and at least two temperatures: +25 and −5°C. At 25°C in four of the five best solvent mixtures (B–E), most of the amide peaks disappeared after the few minutes which were required for sample mixing and cursory locking/shimming. In the case of neat HFIP, loss of amide resonances at 25°C was somewhat slower ($t_{1/2}$ approximately 10 minutes; data not shown). Loss of signal in all cases was due to exchange involving exchangeable deuterons from the protic solvent components in each mixture. Because the freezing point of HFIP is −4°C, we did not attempt to examine exchange in this solvent at −5°C. Attempts to examine TFE:H_2O 1:1 + 0.5% FA at −5°C were hampered by phase separation at the low temperature. How-

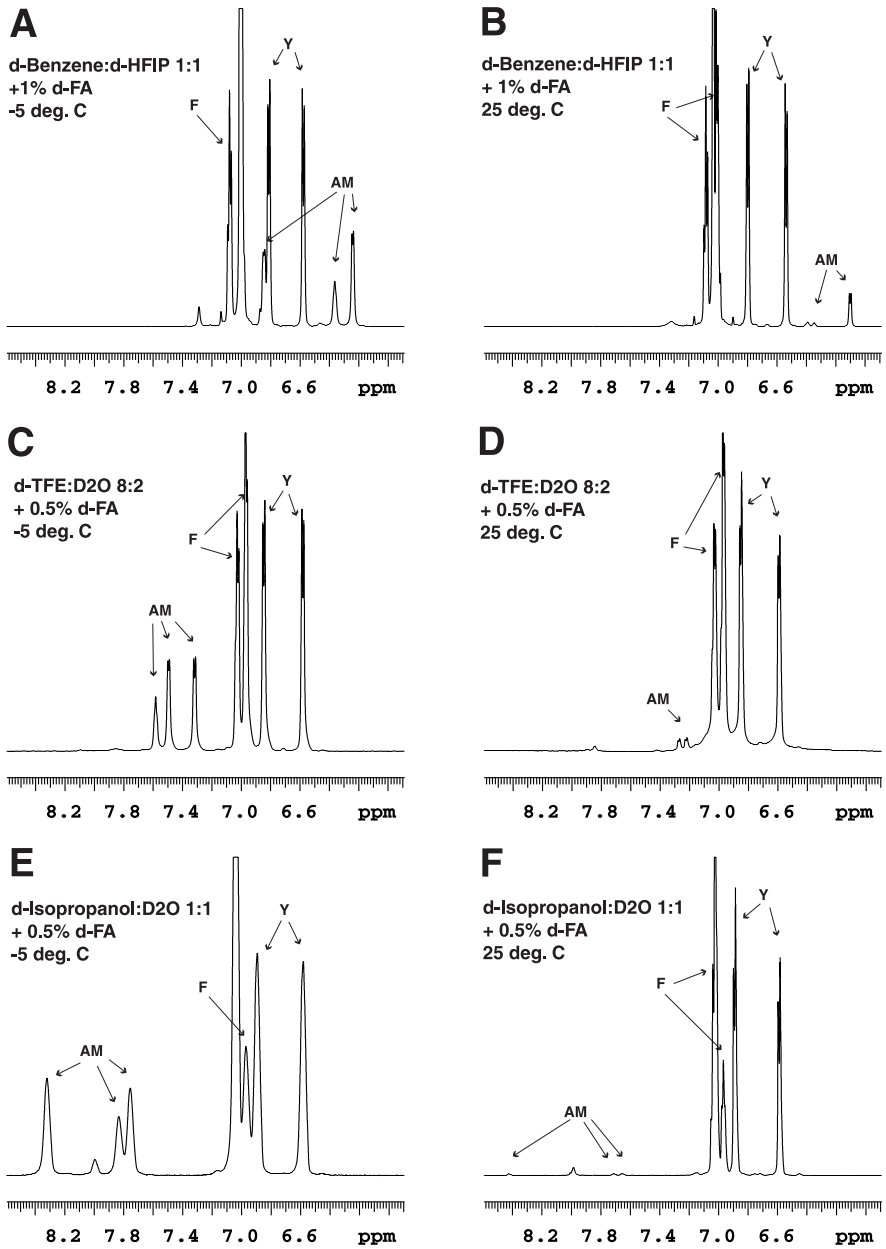

FIG. 3. ^{1}H NMR spectra (amide/aromatic region) of leucine enkephalin (Tyr-Gly-Gly-Phe-Leu) in three different solvent systems obtained 10 min after dissolution at −5 and at +25°C.

ever, when exchange was examined in the other three best solvent mixtures at −5°C (B, C, and E), unwanted amide exchange was observed to be dramatically reduced. Figure 3 presents data from these three solvent systems obtained at −5 and +25°C (six samples) 10 min after sample mixing. Almost complete amide peak intensities are observed in all of the spectra obtained at −5°C. The intensity of the leucine enkephalin amide peaks decreased by, at most, only 5–10% after 1 h of exposure in the three systems represented in Fig. 3 at the depressed temperature (data not shown).

Application of Overall Amide Exchange Scheme to Uniformly Labeled DAGK in Bilayers

Before proceeding to expensive single-site-labeled DAGK samples, we carried out preliminary experiments on uniformly labeled samples. Based on the above solvent screening, the benzene:HFIP 1:1 + 1% formic acid mixture was chosen as the solvent system for implementing and testing the overall AE procedure. U(^{15}N)-DAGK was reconstituted and exposed to D_2O or H_2O (control) followed by freeze-drying, redissolution

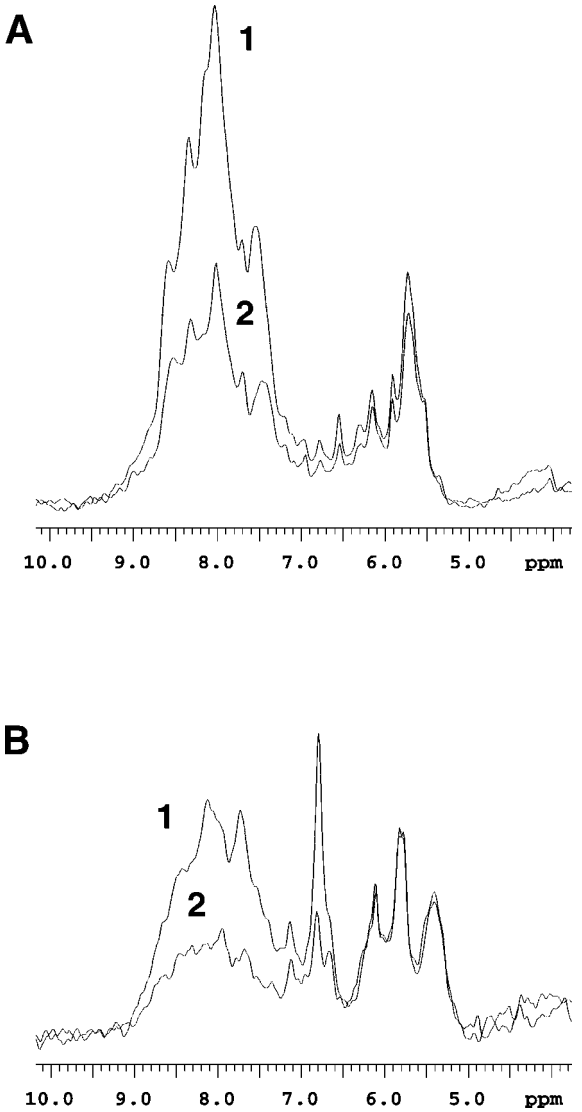

FIG. 4. HSQC spectra of uniformly ^{15}N-labeled DAGK in organic solvent (*d*-benzene:*d*-HFIP 1:1 + 1% *d*-FA) following original exposure of DAGK in lipid bilayers to H_2O (1, control) or D_2O (2) for 1 h. Spectra were obtained on different samples at either room temperature (A, 2048 scans, 30 Hz exponential line broadening) or −5°C (B, 2750 scans, 30 Hz exponential line broadening).

in the organic solvent mixture, and HSQC NMR. Spectra were acquired for samples at both +25 and −5°C (one sample at each temperature). Loss of intensity due to D_2O exposure *in bilayers* could be observed at both cases (Fig. 4). However, there are a number of problems confounding straightforward interpretation of these data in terms of bulk amide exchange. The fact that both control and exchange spectra at 25°C were rather noisy despite a large number of scans suggests that there may have been considerable H–D exchange occurring *in the organic solvent mixture* for both exchange and control samples. This would not lead to spurious measurements (both control and exchange spectra will be equally affected), but does reduce the accuracy of integrated intensity measurements. A more serious problem is reflected by the fact that the true vertical scale for the high-temperature spectra (Fig. 4A) is actually 3 times higher than that of the low-temperature spectra (Fig. 4B). This indicates a considerable loss of signal intensity due to extreme line broadening of many resonances at the lower temperature. Indeed, even in the 25°C case, a number of amide peaks may not be represented because of line broadening so extensive that it results in complete cancellation of antiphase magnetization during the HSQC sequence.

Application of Overall Amide Exchange Scheme to Specifically Labeled DAGK in Bilayers

In the case of DAGK, a route to site-specific exchange measurements was provided by the fact that an appropriate mutant library is available. The laboratory of James Bowie at UCLA has created a series of DAGK mutants, each of which has only 1 cysteine and in which the position of the single cysteine is systematically varied throughout the 121 residue sites of DAGK (unpublished). We biosynthetically labeled a number of these mutants with ^{15}*N*-cysteine, such that each mutant has only a single ^{15}N-labeled site. Since only a single proton in the protein will have an attached ^{15}N resonance, assignment is trivial: a lone peak will ultimately be observed when the scheme of Fig. 1 is implemented.

The full AE procedure was executed on about 30 different specifically labeled DAGK mutants. A variety of results were obtained, with representative examples being presented in Fig. 5. Figures 5A and 5B represent unambiguously positive results, revealing that positions 102 and 113 undergo modest and little amide exchange, respectively, during the 1 h in which bilayer-associated DAGK was exposed to D_2O. The fact that the final spectra are of satisfactory signal-to-noise reflects both the relatively narrow linewidths of the amide protons being observed and an acceptably low degree of post-trap H–D exchange, even at 25°C. This latter observation suggests that these amide sites are protected from solvent when DAGK is solubilized in organic solvent mixtures. Similar results were obtained for a number of the residues of the third transmembrane segment of DAGK (residues 100–120). Figure 5E shows spectra obtained for position 113 at −5°C. Unlike the 25°C case for this site (Fig. 5B), signals cannot be observed due to extreme line broadening of its resonance at the depressed temperature.

A second class of results is represented by Figs. 5C and 5D. In these cases, it is possible to establish that considerable amide exchange occurred during time *t*. However, multiple peaks appear to be present. This observation suggests slow-exchange structural heterogeneity for some regions of the solvent-trapped protein. It should be pointed out that this does not imply structural heterogeneity for the original hydrated vesicular sample. It is, perhaps, unsurprising that a rather complex protein with multiple charged residues may populate

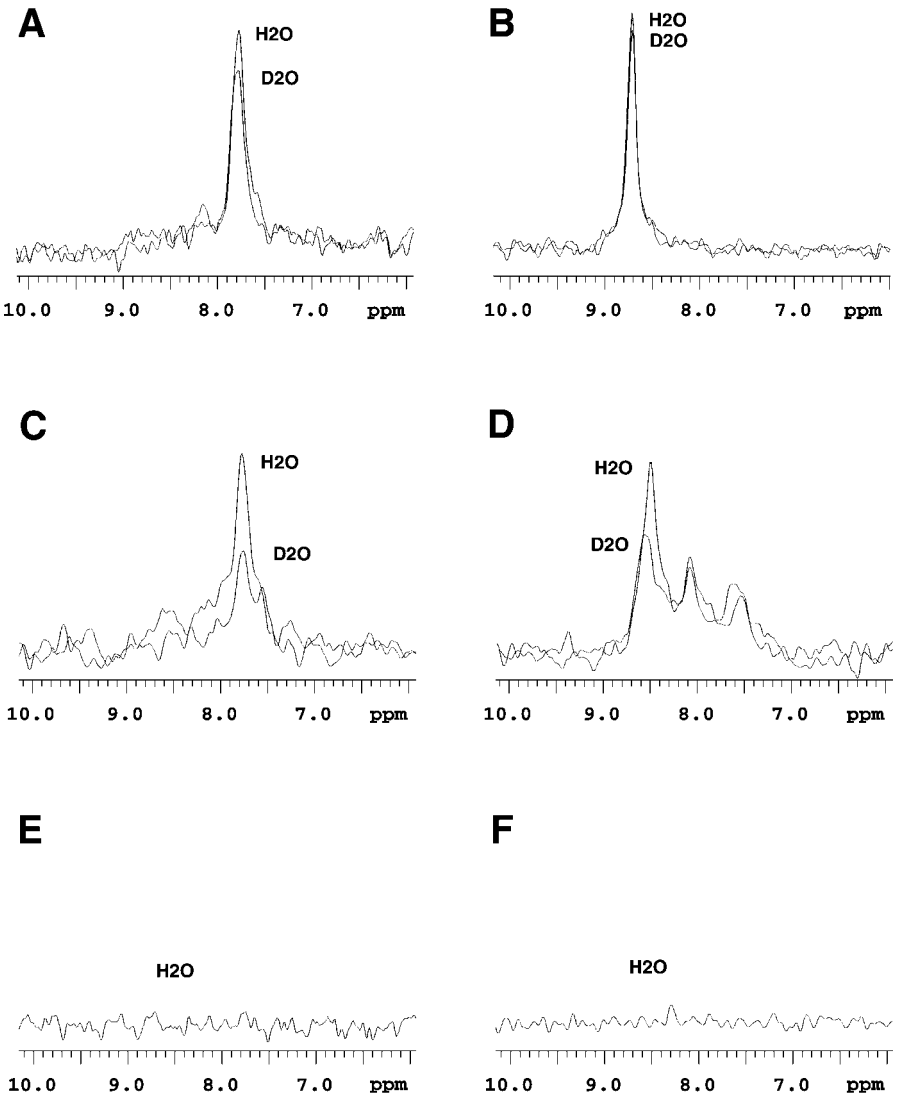

FIG. 5. HSQC spectra of single-(^{15}N)Cys-labeled DAGK mutants in organic solvent (*d*-benzene:*d*-HFIP 1:1 + 1% *d*-FA) following original H–D exchange for 1 h in lipid bilayers: (A) Cys102 mutant at 25°C (20,000 scans); (B) Cys113 mutant at 25°C (15,000 scans); (C) Cys10 mutant at 25°C (8000 scans); (D) Cys53 mutant at 25°C (15,000 scans); (E) same mutant as (B) (Cys113), only spectrum acquired at −5°C (control spectrum only is shown, 2750 scans); (F) Cys1 mutant at −5°C (10,000 scans, control spectrum only). Note that Cys1 is not at the true N-terminal of DAGK: there is a 10 residue purification tag on the N-terminal side of the first position of DAGK's bona fide sequence. These spectra were processed with 20–50 Hz of exponential line broadening being applied.

multiple slowly interchanging conformational states in a uniform low dielectric medium.

Finally, there were some positions where no signal was obtained for either control or exchange samples at either +25 or −5°C (e.g., Figs. 5E and 5F). These represent positions either where the linewidths were too broad to permit an HSQC spectrum to be obtained or where undesired H–D exchange occurring in the organic solvent mixture was complete by the time the HSQC spectrum was recorded (a few minutes after dissolution). This latter explanation likely accounts for the depicted results for position 1 (Fig. 5F). In this case the residue is on the C-terminal end of DAGK's N-terminal polyhistidine purification tag, which is highly mobile and yields very sharp nonamide proton NMR resonances (*25*).

DISCUSSION

In the Results it was demonstrated for DAGK that the method outlined in Fig. 1 allows in-bilayer H–D amide exchange to be monitored at some sites on DAGK, but not at others. Most of the problems encountered in unfavorable cases were related to imperfections in the organic solvent system used to resolubilize the exchanged protein for NMR analysis. Fully aprotic solvents were not found to be effective in solu-

bilizing DAGK, perhaps because some parts of DAGK are highly charged (as for most complex membrane proteins). Because it was necessary to use solvents in which exchangeable protons were present, unwanted post-trap H–D exchange was always a possibility. Indeed, post-trap exchange was in some cases severe enough to prohibit any measurement of the degree of in-bilayer amide exchange at room temperature. In more ideal cases, the amide sites of interest were protected by residual secondary and/or tertiary structure such that this unwanted exchange was not so extensive and useful measurements could be made. Lowering the temperature suppressed unwanted post-trap H–D exchange but generally led to extensive line broadening, often prohibiting observation of HSQC signals. With regard to these difficulties, it is acknowledged that while screening of solvent systems was extensive, it cannot be ruled out that there may be a more optimal solvent system which we simply were not able to find. It should also be pointed out that solvent systems which were not effective for DAGK may work for other membrane proteins, as already demonstrated by Dempsey for much simpler model membrane proteins (*15, 24*).

A second class of problems encountered when the AE method was applied to DAGK is that there appears to be conformational heterogeneity in the organic solvent solubilized form of the protein. In some cases, this was manifested in the form of multiple peaks from a single site, indicative of slow-exchange heterogeneity—a phenomenon which does *not* prohibit AE measurements. However, for some other sites exchange may have been on the intermediate time scale, leading to line broadening extensive enough to prohibit HSQC signals. Again, it should be pointed out that DAGK represents a rather complex example of a membrane protein (three subunits, each with three transmembrane and two highly charged segments). We were unable to find a solvent system which led to retention of a well-defined folded conformation *or* which reduced DAGK to a fully unfolded structural state. For membrane proteins less complex than DAGK but more complex than the single transmembrane span proteins for which most previous NMR-based AE measurements have been made, it may be easier to find appropriate solvent systems which lead to samples with fully folded or fully unfolded protein, such that the heterogeneity problems documented for DAGK do not pertain.

The AE method described in the Results is demanding in the sense that two samples (exchange and control) are required to determine the percentage of amide exchange for a single site at a *single* time point. Given the expense of labeled amino acid such as ^{15}N-cysteine (we paid approximately 750 US dollars per gram), there are limits on how many time points can be taken per site. Thus, the proposed method may often be best employed as an "all or nothing" class of measurement (where only one time point per site is measured). Fortunately, in the case of membrane proteins such measurements may be very useful: water-exposed loops of membrane proteins would normally be expected to undergo complete and rapid exchange, whereas amides buried in the membrane are expected to undergo virtually no exchange, even after long incubation times. Thus, single time point measurements may be very helpful as a way of mapping out the exact sequential locations of loops in membrane proteins—information which is hard to obtain by any method.

Finally, it must be emphasized that the proposed method requires a method for introducing ^{15}N labels site specifically into the membrane protein of interest. This is possible for polypeptides via chemical synthesis. Examples of synthetic libraries of site-specifically labeled membrane polypeptides can be found in the literature (*31, 32*). In the case of larger membrane proteins, a flexible labeling strategy involves starting with a protein which is bereft of one amino acid type. Such a protein can be generated by mutagenically altering all amino acids of the chosen type to something else. This is followed by systematically introducing a single residue of that type throughout the sequence of the protein. Ideally, the protein can then be biosynthetically labeled by protein expression in a medium containing labeled amino acid (*33–35*). In the case of DAGK, such a requirement was fulfilled by the availability of a library of single-cysteine mutants. Cysteine will often be a good choice for studies such as these because it is a relatively rare amino acid type in most proteins, it is a conservative amino acid substitution for most residue types, and the unique chemical reactivity of the thiol moiety lends itself well to a variety of other structural biophysical experimental approaches which can complement the amide exchange measurement (spin labeling for EPR, modification with a fluorophore, etc.; cf. Refs. (*36*) and (*37*)). In addition to DAGK, extensive single-Cys mutant libraries have already been prepared for several other complex membrane proteins, including chemotaxis receptors, a potassium channel, and a transporter, *lac* permease (*36–40*).

CONCLUSIONS

In this paper a method for measuring site-specific amide exchange rates for integral membrane proteins has been presented. While it has limitations, in favorable cases this method should prove useful in characterizing the structures of membrane proteins. Perhaps more importantly, it is hoped that this work will be used as a stepping stone in the development of easier and more robust NMR-based methods for measuring amide exchange rates in the large but difficult family of membrane proteins.

EXPERIMENTAL

Labeling, Purification, and Bilayer Reconstitution of DAGK

E. coli strains containing inducible plasmids for polyHis-tagged DAGK single-cysteine mutants (*41*) were a gift from James Bowie of UCLA. For uniform ^{15}N enrichment of

DAGK, cells were grown in minimal medium, which included $^{15}NH_4Cl$ as the sole nitrogen source (*25, 33, 34*). For specific ^{15}N-Cys enrichment, cells were grown in minimal medium supplemented with unlabeled amino acids plus ^{15}N-Cys (*33, 42*). Labeled cysteine was obtained from Cambridge Isotopes Lab (Andover, MA).

Purification of DAGK was performed as described previously (*25, 27*). In the final elution step, DAGK was eluted from an affinity column with 1% DM plus 0.5 M ammonium hydroxide to yield >95% pure DAGK in micellar DM solution. The eluted DAGK was typically 5–30 mg/ml. DAGK was quantitated based on its absorbance at 280 nm.

To the purified DAGK, mixed micellar solutions of 100 mM 1-palmitoyl-2-oleoylphosphatidylcholine (POPC, Avanti Polar Lipids, Alabaster, AL) and 300 mM *n*-octyl-β-glucoside (Anatrace) were added. The DAGK–detergent–lipid mixtures were then transferred to dialysis tubing (Spectra-Por 1.1, molecular weight cutoff = 8 kDa, Spectrum, Houston, TX) and subjected to dialysis at 4°C against one change of 50 mM phosphate buffer and one change of H_2O (24 h each) to remove detergent, yielding a milky suspension of DAGK-containing multilamellar POPC bilayers (1:100 mol:mol ratio).

For DAGK solubility studies, approximately 20% by weight DAGK in DM powder was prepared by freezing DAGK–DM–ammonium hydroxide mixtures prepared as described above in liquid nitrogen and then lyophilizing.

Amide Exchange, Solvent Trapping, and NMR Determination of the Percentage of Amide Exchange

Reconstituted DAGK in POPC bilayers was usually frozen in liquid N_2 and lyophilized to yield a powdered DAGK–POPC mixture. The powder was divided into exactly equal portions which were then rehydrated with D_2O and H_2O (control) buffers (20 mM Na phosphate, pH 7.0), respectively, and incubated for 1 h at 30°C. Both exchange and control samples were then frozen in liquid N_2 and relyophilized. Both dried samples were dissolved in deuterated organic solvent system and immediately subjected to NMR. Great care was taken to ensure that both D_2O exchange and H_2O control DAGK samples were of identical composition and subjected to exactly the same handling, including careful timing of the time between final dissolution and the start of NMR acquisition.

With the above procedure, there may be concern that the process of hydrating lyophilized lipid–protein mixtures could lead to transient protein structural perturbations which could expose normally exchange-resistant amide sites to facile D_2O exchange during the hydration process. With this concern in mind, we prepared a vesicular U-^{15}N-DAGK sample using ultracentrifugation at 250,000*g* to pellet the DAGK-containing vesicles from protonated buffer solution. As much of the protonated buffer was removed from the pellets as possible and identical samples were then incubated with a large volume excess of a D_2O or H_2O (control) buffer for the H–D exchange period. Following freeze-drying and redissolution of control and exchange samples in acidic benzene/HFIP, the degree of bulk amide exchange was observed to be the same by NMR (within experimental error) as when the standard procedure was employed.

NMR Methods

NMR studies were carried out with a 600 MHz Varian INOVA spectrometer and 5 mm $^1H/^{13}C/^{15}N$ or $^1H/X$ PFG-indirect probes. $^1H–^{15}N$ heteronuclear single quantum coherence (HSQC) spectra were acquired with a gradient-enhanced HSQC pulse sequence (*43*). Both D_2O- and H_2O-exposed samples were run under exactly the same conditions, using the same parameters and same number of transients. Quantitation of the percentage of amide exchange was obtained by comparing HSQC from exchanged and control samples. Calculation of the peak areas was performed with standard Varian routines.

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