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Microdrop screening: A rapid method to optimize solvent conditions for NMR spectroscopy of proteins

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Abstract

Determining appropriate solvent conditions is a crucial first step for carrying out NMR spectroscopy of proteins, but rapid and efficient methods for doing so are currently lacking. Microdrop screening examines a large number of different solvent conditions using very small amounts of protein and minimal labor. Starting from one initial buffer condition, small aliquots of protein solution are combined with an array of solutions in which concentration, pH, buffer type, and added stabilizers are systematically varied. The protein concentration of each microliter-sized test drop ('microdrop') is gradually changed using vapor diffusion, and the solubility of the protein is determined by visual examination. A variety of analytical techniques may be applied to the contents of the microdrops to monitor enzymatic activity, aggregation, ligand binding, and protein folding.

Abbreviations: Bicine, [N,N-*bis*-(2-hydroxyethyl)-glycine]; BME, beta-mercaptoethanol; BOG, *n*-hexyl-b-D-glucopyranside; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; DMSO, dimethylsulfoxide; DTT, dithiothreitol; GMF-β, glia maturation factor beta; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; MES, [2-(N-morpholino)ethanesulfonic acid]; MOPS, [3-(N-morpholino) propanesulfonic acid]; PIPES, [piperazine-N,N′-*bis*-)2-ethanesulfonic acid]; TES, (2-[tris-(hydroxymethyl)-methyl]-amino-ethanesulfonic acid); TFE, trifluoroethanol; TMAO, trimethylamine-N-oxide; TRIS, [*tris*-(hydroxymethyl)-aminomethane].

Introduction

Recent advances in hardware and techniques have greatly advanced the ability of NMR spectroscopy to provide high resolution, three dimensional structures of proteins in solution. Advances in the methods that support these studies, such as molecular biology, protein expression, and computational techniques, have further enhanced the ability of NMR spectroscopy to provide structural information, particularly for proteins of greater than 15 kDa molecular weight.

Despite these advances, limited protein solubility and stability frequently derail NMR studies. In the future, this problem is likely to become more common as spectroscopists attempt to extend the size of proteins for which NMR can provide structural information, e.g. by using truncated constructs or sub-domains of larger proteins, which suffer from aggregation and misfolding more frequently than do native proteins.

In the area of sample preparation, NMR is roughly at the point where crystallography was 20 years ago. The main approach for identifying solvent conditions for NMR studies currently consists of exchanging the protein into various buffers, then concentrating the solutions and assessing the solubility. Once a buffer and pH have been identified in which the protein is reasonably stable, an empirical approach is taken in varying the use of such additives as salt, reducing agents, glycerol, detergents, etc. in order to maximize solubility and stability. The process of transferring the protein into various buffers is tedious, time-consuming, and difficult to carry out on a very small scale, and properly sampling all of the possible combinations

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of variables consumes large amounts of valuable protein. A recently reported method using microdialysis buttons (Bagby et al., 1997) uses much less protein than the conventional approach, but we find it to be somewhat inconvenient because extensive manipulations are required to prepare each button, and only one protein concentration can be tested per button.

While spectroscopists seek to solubilize their proteins, X-ray crystallographers have long contended with the converse problem in identifying conditions under which to precipitate proteins out of solution in order to grow crystals. Their answer to this problem has been to apply the technique of vapor diffusion (reviewed by McPherson, 1990; Ducruix and Giegé, 1992) to carry out controlled precipitation. To use this method, a protein solution is typically combined with a precipitating agent and the mixture is sealed within a chamber containing a solvent reservoir in such a way that solvent is gradually drawn out of the protein solution, leading to supersaturation and precipitation of the protein crystal. Because it is intended to induce controlled precipitation, the crystallographic method is obviously unsuitable for preparing NMR samples. Although the solubility trends observed in crystal trials will sometimes suggest conditions for NMR samples, the results cannot be reliably extrapolated because precipitants perturb protein/solvent interactions. In addition, precipitants often interfere with activity assays and spectroscopic measurements, making it impossible to ascertain if the protein in the drop exists in its functional form.

In this paper, we have modified the vapor diffusion method for the purpose of rapidly and efficiently screening solvent conditions for NMR samples. The key principle of our method is that screening with stabilizers instead of precipitants allows one to optimize *solubility* rather than *precipitation.*

There are several crystallization procedures that may be easily adopted for use with NMR samples. This paper emphasizes the 'hanging drop' technique, which employs 24-well 'Linbro' plates made of transparent plastic. In this method, drops of protein solution are placed on glass cover slips, and each drop is then mixed with a different buffer. The cover slips are then inverted and sealed onto wells containing a reservoir of the corresponding buffer. The protein concentration of the drops gradually increases as water diffuses from the drop to the reservoir. The solubility of the protein in each buffer is then judged by examining the drops for precipitate.

In the following sections, we describe the successful application of the hanging drop technique to a common problem: insufficient solubility of a protein under published buffer conditions. In addition, we provide general strategies for solving protein solubility problems using our screening method.

Materials and methods

^{15}N labeled human recombinant GMF-β with an amino-terminal His_6 tag (total MW ca. 24 kDa) was expressed in *E. coli* using minimal media and purified using metal affinity resin at a yield of 30 mg purified protein per liter media (S. Chambers, J. Fulghum and C. Lepre, unpublished results). The protein was refolded and exchanged into 50 mM potassium phosphate buffer at pH 7.4 as described by Kaplan et al. (1991). Prior to solvent optimization, the solubility limit was 8 mg/ml (330 μm).

After exchange into 10 mM potassium phosphate buffer, a pH screen was carried out using 24 buffers (Table 1). To simplify the problem of testing a large number of possible buffers, a sparse matrix approach was used (Jancarik and Kim, 1991) in that the 24 buffers chosen were heavily biased toward conditions shown to be successful in previous NMR studies. The buffers were at concentrations of 100 mM so that the final pH of the drops would reach the target values even when the buffers were added at ratios below 1:1.

The pH screen was set up using the hanging drop method with a 24-well tissue culture ('Linbro') plate and siliconized glass cover slips (Hampton Research). To prepare the screen, 1 ml of 100 mM buffer was pipetted into each reservoir. Then, 2 μl aliquots of protein solution in starting buffer were pipetted onto the glass cover slips. To each drop was added 1 μl of 100 mM reservoir buffer, and the solutions were mixed by gently drawing and expelling the solution in the pipette tip. The glass slips were then inverted and sealed onto the wells using petroleum jelly. A total of 0.384 mg of protein was used.

The plate was allowed to rest undisturbed at room temperature so that vapor diffusion could take place. The amount of precipitate in the drops was measured by placing the tray against a black background, illuminating it from the side, and visually examining each drop under a microscope. Under these lighting conditions, precipitate appears as a white spot against the black background, and is scored based on the fraction of the drop covered by precipitate (scale of

Table 1. Microdrop pH screen of GMF-β

drop	buffer, pH	score[b]	drop	buffer, pH	score
1	potassium phosphate[a], 5.0	2	13	ammonium acetate, 7.3	2
2	potassium phosphate, 6.0	1.5	14	imidazole, 8.0	3
3	potassium phosphate, 7.0	1	15	bicine, 8.5	3
4	potassium phosphate, 7.4	1	16	bicine, 9.0	4
5	sodium phosphate, 5.5	2.5	17	MES, 5.8	3
6	sodium phosphate, 6.5	0.5	18	MES, 6.2	2.5
7	sodium phosphate, 7.5	0.5	19	MES, 6.5	2
8	sodium acetate, 4.5	3	20	HEPES, 7.0	1
9	sodium citrate, 4.7	2.5	21	HEPES, 8.0	1.5
10	sodium acetate, 5.0	4	22	TRIS, 7.5	1
11	sodium citrate, 5.5	1.5	23	TRIS, 8.0	1.5
12	cacodylic acid, 6.5	1.5	24	TRIS, 8.5	3

[a] All buffers are 100 mM.
[b] Score is based on the surface area of the drop covered by precipitate after 24 hr at room temperature (0 = clear, 4 = entire drop).

0 to 4, with 0 for no precipitate and 4 for precipitate completely covering the drop; see Figure 1). The relative stability of the protein in the drops was assessed throughout the equilibration process by scoring the drops at 12–24 hr intervals for several days. The most distinct pattern of relative stabilities emerged after 24 hrs (Table 1), although the drops probably had not yet reached their final equilibrium concentrations (ca. 16 mg protein/ml) at this time point.

The stabilizer screen was carried out using the most solubilizing buffer conditions identified in the pH screen (sodium phosphate at pH 7.5 and HEPES at pH 7.0) and common, NMR-compatible stabilizers. 24 drops were screened, each containing 2 μl of GMF-β (9.9 mg/ml in 10 mM potassium phosphate) combined with 2 μl of well buffer. Variables tested were: sodium phosphate concentration (25, 50 and 100 mM) and addition of salt (25, 50 and 100 mM NaCl), BME (10 mM), glycerol (5%, 10%), or CHAPS (2 mM).

Results

Two hanging drop screens (pH and stabilizers) were carried out on GMF-β, and these yielded the following results. In the pH screen, the protein was more soluble in sodium phosphate at pH 7.5 than in the original potassium phosphate buffer (score of 0.5 vs. 1.0 after 24 hr, Table 1). In addition, HEPES at pH 7.0 and TRIS at pH 7.5 were good low ionic strength alternative buffers (scores of 1.0). Lastly, MES, acetate, and any buffer with pH $\geq$ 8.5 gave poor solubility (scores of 2 to 4).

The results of the stabilizer screen are summarized below. Only addition of detergent improved solubility; addition of reductant or glycerol and variation of ionic strength either reduced solubility or had no effect.

Conditions screened	*Results*
25 mM, 50 mM, 100 mM sodium phosphate	all concentrations the same
HEPES at pH 7.0	less soluble than phosphate
10 mM beta-mercaptoethanol	no improvement in solubility
5% and 10% glycerol	no improvement in solubility
25 mM, 50 mM, 100 mM sodium chloride	no improvement in solubility
2 mM CHAPS (CMC = 6 to 10 mM)	improved solubility

Based upon the combined results of the two screens, a new sample of GMF-24 was prepared in 50 mM sodium phosphate at pH 7.4 with 2 mM CHAPS. This sample was successfully concentrated to 1.3 mM and used for NMR spectroscopy. Thus, by using microdrop screening it was possible to test 48 different solvent conditions in only two days using less than 1 mg of protein, with the net result that the protein solubility was increased by a factor of four.

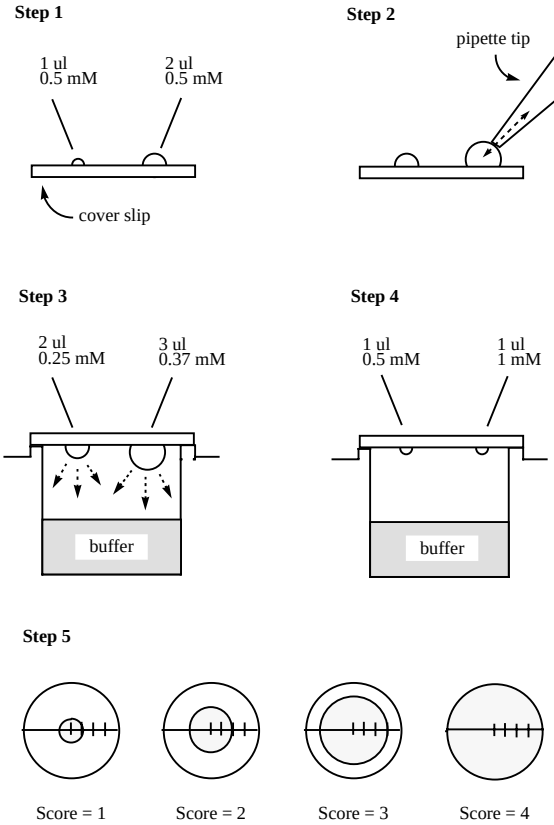

Figure 1. Procedure for preparing a typical screen using a 24-well Linbro plate. *Step 1*: Pipette protein dissolved in starting buffer onto cover slip (drop volume and protein concentration are indicated). Two drops are prepared per slip in order to test two different final concentrations. *Step 2*: Mix protein drops with 1 μl volumes of reservoir buffer by drawing and expelling solution into pipette tip. *Step 3*: Invert cover slip and seal onto buffer reservoir. *Step 4*: Allow plate to rest undisturbed until vapor equilibration is complete. *Step 5*: Score drops by estimating the surface area of the drop covered by precipitate, as viewed from above. Outer circle depicts the diameter of the drop, inner circle (shaded) depicts precipitate.

Discussion

This section discusses general strategies for solving protein solubility problems by microdrop screening. Basically, two approaches may be followed: diffuse solvent out of the drop (concentration) or diffuse solvent into the drop (dilution). The same principle underlies both approaches: start with the protein in a solution in which it is soluble, and slowly change the solvent conditions in the presence of various potential stabilizing agents. If a given stabilizer is effective, then the protein will remain soluble as the solvent conditions change to a point where the protein would otherwise precipitate.

Concentration method

The idea behind the concentration approach is to start with the protein dissolved in starting buffer at a concentration close to its solubility limit, then mix it with small drops of various buffers and stabilizers, and allow the drops to equilibrate with a reservoir containing a higher solute concentration. As solvent diffuses out of the drop, the protein concentration will rise above the value at which it would otherwise precipitate, and only those drops containing additives that solubilize the protein will remain clear.

When a new protein is purified for the first time, it is typically dissolved at low concentration in a starting buffer that is known to be fairly innocuous. In addition, various putative stabilizers are usually present, such as salt, detergent, glycerol, and excess reductant. The goal of the first screen is to identify buffer conditions for concentrating the protein without precipitation, preferably while avoiding reagents that interfere with NMR studies. For this reason, preference is given to buffers and stabilizers that are aprotic or commer-

cially available in deuterated form. Other factors (long term stability, linewidths, etc.) can be dealt with in follow-up screens.

The protein is prepared for screening by dissolving it in the starting buffer at the lowest buffer concentration sufficient to maintain pH control (ca. 5 to 10 mM), then concentrating it as much as its solubility will permit. In our experience, a protein can often be brought to concentrations sufficient for NMR studies without immediately precipitating, but it then gradually precipitates out of solution over the next 12 to 24 h. Such proteins are excellent candidates for microdrop screening.

For proteins with very poor solubility, it may be necessary to initially screen with a dilute sample, then rescreen the most promising conditions again at higher concentration. Since the protein can be concentrated by up to approximately four-fold by reducing the amount of equilibration buffer added to the drop, it isn't necessary to concentrate the original solution to the final concentration desired for NMR studies. In fact, by replacing the buffer in the reservoir with more concentrated solution, it is possible to concentrate the drop by a large factor through a series of controlled steps. The ability to concentrate the protein *in situ* during the screen makes microdrop screening more flexible than dialysis-based methods, such as the button test (Bagby et al., 1997).

If necessary, stabilizers can be included in the starting solution and then systematically removed to test their importance in later screens. If it is particularly difficult to find initial buffer conditions to purify a new protein, then a microdrop screen may be run directly on the spun cell lysate, and the choice of pH and buffer made using an activity assay.

Figure 2 illustrates a stepwise, multiscreen approach used to systematically converge upon solvent conditions. The first microdrop screen varies pH and buffer type, since the buffer choice dictates the conditions used for the subsequent stabilizer screens. For the hanging drop method, a single 24-well plate is sufficient for a screen from pH 3 to 9, with most data points clustered in the optimal range for NMR studies (between pH 5 and 8).

Solvent will diffuse out of the drop until its ionic strength is approximately the same as the reservoir buffer. The concentration of protein in the drop at equilibrium is thus controlled by the ratio of the protein and reservoir solutions in the drop. For example, if two volumes of protein solution are combined with one volume of reservoir solution, then the concentration of protein in the drop after vapor diffusion will be approximately twice that of the starting protein solution, although the precise composition of the final drop will differ slightly from the reservoir due to the presence of protein and starting buffer in the drop. Several different concentrations may be tested per well by spotting several drops with different ratios of protein to buffer on the same cover slip; up to four drops can be spotted per slip, depending on the manual dexterity of the practitioner. A typical 24-well screen using 1 to 2 μl drops of 10 mg/ml protein solution per well consumes only 0.24 to 0.48 mg of protein.

If the protein is soluble over a broad pH range, making it difficult to identify the best value, then a 'torture test' may be carried out by slowly increasing the temperature: the less stable or soluble samples will precipitate first. On the other hand, if the protein is insoluble throughout the pH range studied, then the screen may be rerun with common stabilizers (salt, reducing agent, glycerol) present, or at low temperature.

Once pH and buffer conditions have been chosen, further improvements in solubility, stability, etc. may be attempted by screening putative stabilizing agents. The stabilizers most likely to succeed are tried first, followed by those that are less common and more likely to interfere with spectroscopy. Stabilizer concentrations should be kept low at first in order to avoid potential problems: e.g. salt concentrations above 100 mM may result in Rf heating, a large excess of reductant may reduce structurally important disulfide bonds, glycerol viscosity effects may induce unacceptable line broadening, and detergents may form micelles above their CMC. Due to the practical limits of deuterium incorporation, even deuterated reagents may introduce intense proton signals at high concentration (e.g., 10% glycerol added as a stabilizer will contribute a 55 mM methylene proton signal even when 99% deuterated). On the other hand, if the initial pH screen was run with NMR-incompatible stabilizers present, then at this stage one may try to systematically remove or replace them.

If any additives in the first screen give improved solubility or stability, it is worthwhile to extend those conditions before trying new additives, e.g. try higher concentrations, a combination of stabilizers, or a different temperature. If one detergent is found to have a positive effect, then other common detergents (CHAPS, Triton, BRIJ, Nonidet, etc.) may be tried. Kits containing various detergent solutions intended

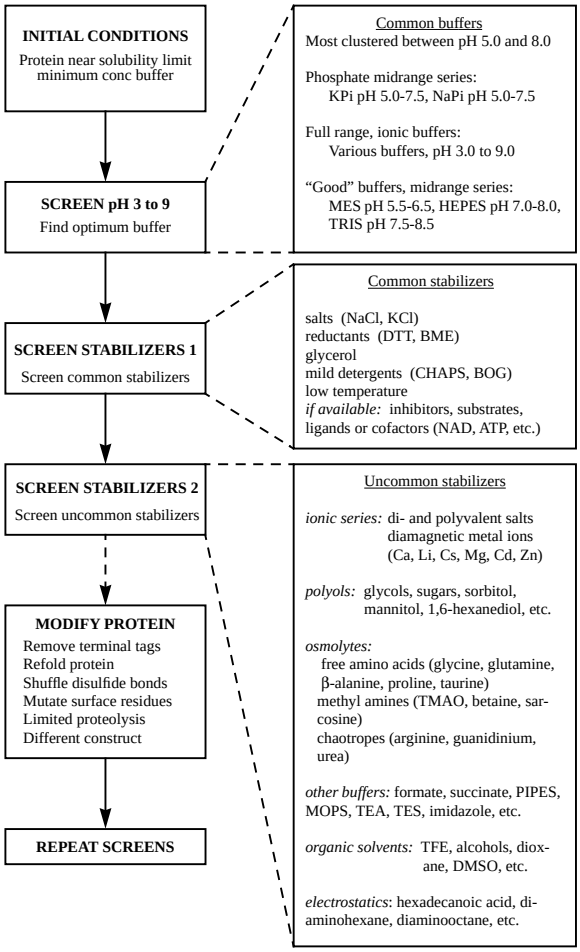

Figure 2. Flowchart depicting the microdrop screening process.

for use in crystallizations are commercially available (Hampton Research, Inc).

The exotic stabilizers are left for last (Figure 2). Of these, the osmolytes have proven to be the most effective, although they can introduce intense signals and sample heating problems because concentrations up to several molar may be required (Yancey et al., 1982; Santoro et al., 1992; Matthews and Leatherbarrow, 1993). Unlike the relatively benign stabilizers used in earlier screens, some of the reagents on this list are quite likely to denature the protein or alter secondary structure, and precautions should be taken to ensure that the protein retains its functionality.

If problems remain after screening all of the conditions that ingenuity can devise, then it may be necessary to try modifying the protein by removing carboxy- and amino-terminal tags, refolding (especially in the presence of possible cofactors and metal ions), reshuffling disulfide bonds (using redox reagents or disulfide isomerase), limited proteolysis, site-directed mutagenesis, or expression of alternative constructs.

In addition to the simple solubility test, a wide variety of other tests may be performed on microdrops in order to provide additional criteria for selecting solvent conditions based on the activity, aggregation state, and folding state of the protein, for example:

assays: functional activity, immuno-reactivity, protein concentration. optical methods: UV/vis/IR spectroscopy, CD, fluorescence, light scattering.

other methods: HPLC/FPLC, gel electrophoresis, terminal sequencing analysis, mass spectrometry, micro-scale NMR spectroscopy.

Some of these analyses require more than a few microliters of solution. In order to provide a larger

sample, vapor diffusion may be carried out using the 'sitting drop' method (McPherson, 1990; Ducruix and Giegé, 1992). In this method, drops of protein solution are placed in individual depressions in a multi-well plate, or on small bridges placed in the wells of a 'Linbro' plate, allowing drop volumes of up to several hunded microliters to be used. By judicious choice of which analyses are performed in what order, it is feasible to measure all of the key indicators of the protein state (solubility, activity, monodispersity, and folding state) using only the contents of a single microdrop. For example, if a microdrop screen is set up using 20–30 μl sitting drops, then from those drops in which the protein is soluble a ≤ 1 μl aliquot may be removed for an activity assay. For those drops which show activity, the remainder may be analyzed in a dynamic light scattering instrument to determine aggregation state. For those samples which are monodisperse, the solution may be transferred to a micro-scale NMR tube and a test spectrum acquired.

Dilution method

Prior to optimization of the solvent conditions, a target protein may be almost completely insoluble unless unfolded in chaotrope solution or in the presence of high concentrations of stabilizers that are undesirable for NMR studies. In this case, solvent conditions may be screened by diffusing solvent into the drop: the protein is concentrated with chaotrope or stabilizer present, then mixed with buffer containing a new stabilizer and allowed to equilibrate with a dilute reservoir. As solvent diffuses into the drop, the chaotrope/stabilizer concentration will drop below the level normally needed to keep the protein soluble, and only those drops containing new additives that stabilize the protein will remain clear. Of course, the protein concentration will also decrease; if this presents a problem then another method (such as the button method of Bagby et al.) may be preferable.

The dilution principle may be applied in a batch screening method by using a 96-well microtiter plate. In this case, the plate is placed in a sandwich box and all wells are allowed to equilibrate against a common solution. An advantage of using microtiter plates is that automated systems for running assays, etc. are readily available. In an alternative application of batch screening, small amounts of a volatile acid or base is added to the reservoir. The pH of the microdrops then increases or decreases gradually as the acid or base diffuses from the reservoir.

Conclusions

The microdrop screening method has proven to be an effective tool for optimizing NMR sample conditions for several different proteins. Because the screen uses a uniform initial condition, it eliminates the need to prepare batches of protein in a variety of different buffers. Thus, a large number of sample conditions may be rapidly tested using sub-milligram amounts of protein, while readily providing critical information about the protein under the same conditions as exist in the NMR tube. This efficiency permits screening of a wider variety of conditions, and subtle combinations of conditions, than is possible using conventional procedures. Overall, microdrop screening increases the efficiency of the search for solvent conditions (defined as the number of sample conditions studied per unit amount of protein) by approximately an order of magnitude over trial-and-error methods.

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