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Communication

Increasing the sensitivity of cryoprobe protein NMR experiments by using the sole low-conductivity arginine glutamate salt

Guillaume M. Hautbergue [a], Alexander P. Golovanov [b,*]

[a] Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK
[b] Faculty of Life Sciences and Manchester Interdisciplinary Biocentre, The University of Manchester, 131 Princess Street, Manchester M1 7DN, UK

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Abstract

Decrease in experimental sensitivity of cryoprobe experiments for salty samples, attributed to increased sample conductivity, has been a long-standing issue in protein NMR. Salt concentration can not be simply reduced as this often leads to protein aggregation. A simple and inexpensive solution to this problem is demonstrated here. We show that even for proteins prone to aggregation, the traditional solubilizing salt, 100 mM NaCl, can be completely replaced by 50 mM L-Arg and L-Glu. This replacement simultaneously reduces the sample conductivity and improves protein solubility. Up to a 6-fold overall increase in experimental sensitivity was achieved, in comparison with the traditional salty buffer. At constant protein concentration up to 2-fold increase in sensitivity was observed. The lengths of the proton $\pi/2$ pulses were also significantly decreased, up to the level typical for non-salty samples in water.

Keywords: NMR Cryoprobe; Protein solubility; Sensitivity; Salt

1. Introduction

Cryogenically-cooled probes have proven to be a very useful new tool in solution-state NMR studies of molecules. Significant gains in sensitivity (up to 3- to 6-fold) can be achieved by cooling the detecting coil and preamplifier, reducing thermal noise [1,2]. However, the presence of salts in NMR samples significantly diminishes these sensitivity gains [2–5]. The nature of this effect is attributed to the increased ionic and dielectric conductivity of the sample, which leads to dissipation of the RF power and appearance of ring currents in the sample tube [2–6]. High salt concentrations also lead to sample heating, increase in measured pulse lengths and, in extreme cases, may even cause difficulties tuning the probe [5]. At very high salt concentrations the potential sensitivity gains offered by the cryoprobes may be lost altogether. The problem with loss of sensitivity becomes even more severe at high fields [7]. The previous studies identified that the increased noise from the sample mostly originates from the particular "RF hot spot" areas at the parallel edges of the sample cavity [6,8]. Removing sample from those areas by using thinner diameter [5] or flat sample tubes can lead to significant reduction of noise and shortening the pulse length [6,8]. Using such tubes however leads to reduction in sample volume. For samples that are concentration-limited, such as many proteins, using less volume in turn reduces the overall experimental sensitivity. As the result, for flat sample tubes a compromise is reached between decreasing noise by removing sample from certain areas, and decreasing signal intensities because of reduction in sample volume and quantity. Overall, the usage of flat tubes leads to a modest re-gain in sensitivity of ca. 45% [6,8].

Typically, protein sample solutions contain significant amounts of two different kinds of salts. They are added as a buffer (for maintaining the desired pH), and as a solubilizing agents to prevent protein aggregation. The best

* Corresponding author. Fax: +44 161 306 5201.
E-mail address: a.golovanov@manchester.ac.uk (A.P. Golovanov).

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buffers for use with the cryoprobes are those with the least conductivity and ion mobility [4]. The particular buffer salts used are usually not a critical factor affecting protein solubility, as long as pH optimum for solubility is maintained. Concentration of buffer salts, and hence their contribution to the sample conductivity, can be easily reduced (e.g., to <20 mM) at the small expense of buffering capacity. The main sensitivity losses in the protein NMR experiments performed using cryoprobes originate from the essential *solubilizing* salt, typically $\geqslant$100 mM NaCl. (For consistency, the specific buffers that are essential for protein solubility also should be classified as "solubilizing salts"). The concentration of this solubilizing salt component usually cannot be reduced, as this will in turn reduce the amount of soluble protein, causing loss in experimental sensitivity. The overall sensitivity of cryoprobe protein NMR experiments is thus determined by *both* conductivity and solubilizing property of this essential component, and this is likely to be a trade-off. These two factors therefore should be taken into account together when deciding which solubilizing salt is better for the cryoprobe experiments. Previous studies [4,5,9] have used extremely soluble proteins or constant protein concentrations for comparisons of effects of different salts on sensitivity. In those conditions only the "conductivity" contribution of salt to the overall experimental sensitivity was explored, but not the "solubilizing" contribution. Therefore the practical advantages of suggested additives [4,5,9] for maximizing the overall sensitivity were not clear, as opposed to obvious alternatives such as using just de-ionized water, less salt or simply adding more protein to the sample.

Recently we have demonstrated that the simultaneous addition of 50 mM L-Arg and 50 mM L-Glu (Arg+Glu) to the sample solution, in combination with 100 mM NaCl, dramatically increases maximal achievable protein concentration, and improves the long-term sample stability against precipitation and degradation [10]. Being charged oppositely in the pH range 5–7 which is used widely for protein studies, these amino acids form together a highly-soluble salt. The efficiency and anti-aggregational effect of arginine glutamate has been confirmed in several independent studies [11–14], and the number of protein structures obtained in these conditions is steadily growing (e.g., see Refs [12,14–17]) suggesting that these additives may be as universal as traditional NaCl, but more efficient. It has been established that these amino acids work best only when they are added together [10,11], and thus they provide the most efficient suppression of aggregation (per mole added) compared with other typical additives [11]. The effect of Arg+Glu on the cryoprobe sensitivity has not yet been explored. Here we demonstrate that by using arginine glutamate salt as a sole additive for protein solubilization, it is possible to increase dramatically the overall sensitivity (signal-to-noise ratio) of protein NMR experiments performed on cryoprobes, without using salt-tolerant probes or special sample tubes.

2. Results and discussion

To test the effects of arginine glutamate salt on protein solubility and cryoprobe sensitivity, we conducted NMR experiments for two proteins heavily prone to aggregation, REF2-I(1-155) [15] and SF2(107-196) H183A mutant [17]. These proteins are not soluble in de-ionized water, but have limited solubility at higher ionic strength (for example, 100 mM NaCl). Arg+Glu salt (50 mM) was added to protein preparations, either with or without 100 mM NaCl, and these protein samples were compared to separate samples solubilized using only the 'traditional' NaCl salt. First, we found that it is possible to concentrate both "difficult" proteins *without* any NaCl, using instead just 50 mM Arg+Glu, pH 6.4. The proteins were concentrated in this solution by ultrafiltration up to their solubility limit, 0.7 and 2.6 mM for REF2-I and SF2, respectively. The spectra acquired for these samples have the highest sensitivity among various conditions tested here (Fig. 1, and Table 1). In 50 mM Arg+Glu solution without NaCl, the measured proton 90° pulse lengths were only a fraction (5–9%) longer than those typically measured in de-ionized water (Table 1). Addition of defined amounts of dry NaCl (up to the final concentration of 100 mM) to the same protein samples which were obtained with sole 50 mM Arg+Glu, resulted, as expected, in a significant (up to 2-fold) drop in sensitivity, although the protein concentrations in the samples were the same (Fig. 1 and Table 1). In line with the increased conductivity, the proton 90° pulse measured at constant transmitter power increased dramatically, by about 40-50% (Table 1). Apart from the different sensitivity, the overall quality of the 2D HSQC spectra obtained in the presence of Arg+Glu with or without NaCl was good, with the differences between the signal chemical shifts being small (Fig. 2). In a separate experiment, both proteins were also concentrated by ultrafiltration *up to their solubility limit* in the "traditional" solution of 100 mM NaCl with pH stabilized by 20 mM sodium phosphate buffer (no Arg+Glu). Significant amounts of proteins aggregated and precipitated during this process. The achieved maximum concentrations of soluble material were much lower, 0.15 and 0.7 mM for REF2-I and SF2, respectively. As expected, 90° pulse lengths for these samples were typical for the salty solutions (Table 1). The spectra obtained in this traditional solution were up to 5–6 times less sensitive than those obtained in sole 50 mM Arg+Glu, because of a combination of reduced protein solubility and higher sample conductivity. For SF2, the reduced sensitivity did not affect much the 2D HSQC spectral pattern (Fig. 2) but, for REF2-I, very low sensitivity caused lack of observation of many signals. This comparison of fingerprint spectra obtained in different solvent conditions shows that protein structure was unaffected when using sole 50 mM Arg+Glu. The disappearance of some of the signals in the spectra collected in the absence of Arg+Glu can be explained by the increased protein aggregation at concentrations close to the limit. In the absence of Arg+Glu the

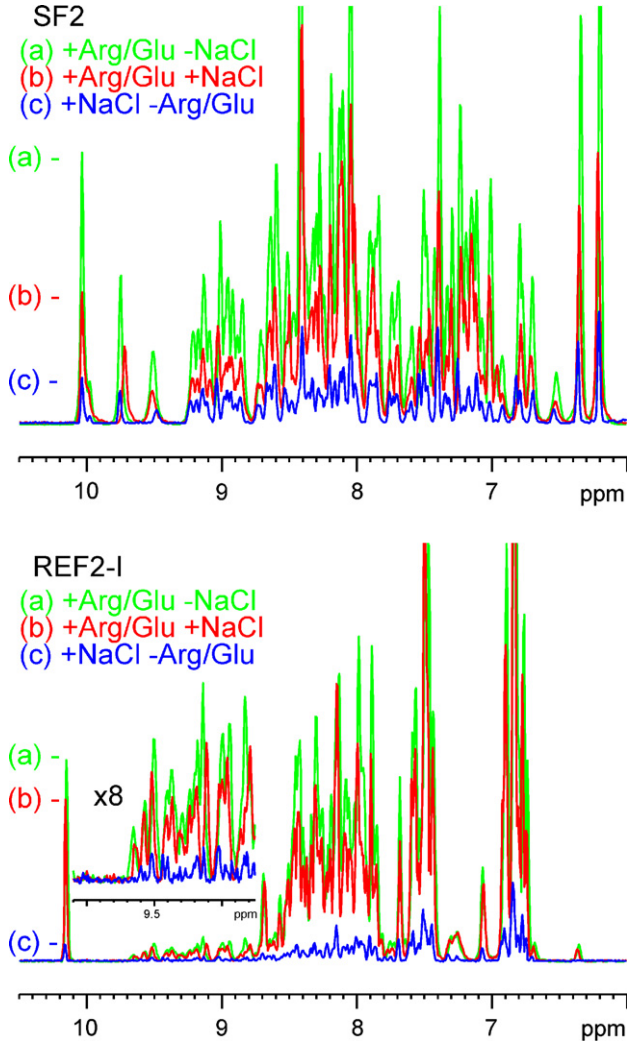

Fig. 1. Horizontal ^{1}H projections of ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled proteins SF2 (top panel) and REF2-I (bottom panel) concentrated and acquired in different solutions. (a) Samples concentrated to the solubility limit in 50 mM L-Arg, 50 mM L-Glu. (b) As in (a) but with dry NaCl added to the same samples to the final 100 mM concentration. (c) Protein samples concentrated in 100 mM NaCl, 20 mM phosphate buffer. All solutions had pH 6.4 and additionally contained 5 mM β-mercaptoethanol and 1 mM DTT. Proteins were concentrated by ultrafiltration using Amicon protein concentrator. In the absence of L-Arg and L-Glu, significant amount of protein aggregated and precipitated during this process. The inset shows enlarged part of the REF2-I spectrum. All the measurements were conducted in a standard 5 mm NMR tube, on a Bruker 600 MHz spectrometer equipped with 5 mm TXI Cryoprobe. The peaks of the highest intensities are clipped. (For interpretation of the references to color, the reader is referred to the web version of this paper.)

proteins also continued to precipitate with time in the sample tubes. Overall, sole usage of Arg+Glu resulted in a significant gain in sensitivity compared to a traditional buffer containing 100 mM NaCl, by favorably combining better protein solubility with lower conductance of the solution.

When the ionic strength increases, the balance between increased protein solubility and ionic conductivity affecting experimental sensitivity becomes very delicate. It should be noted that the combined use of 50 mM Arg+Glu with 100 mM NaCl may increase the protein solubility limit approximately twice, in comparison with using Arg+Glu alone ([10]; Hautbergue, Golovanov, unpublished observations). However, for cryoprobes, this increase in protein concentration is counterbalanced by the sensitivity losses due to the increased solution conductivity. As a result, despite using twice the amount of protein material in the sample, the overall sensitivity of cryoprobe experiments is not noticeably improved. Moreover, the pulses become longer and RF dissipation increases, causing additional sample heating. It should be also noted that whereas adding 100 mM NaCl to sample solution caused 2-fold drop in sensitivity for SF2, the corresponding drop for REF2-I was lower, only 1.47-fold (Table 1). The expected drop in experimental sensitivity due to increase in sample conductivity should be however the same [2–5]. We explain this lower drop for REF2-I by small decrease in its aggregation leading to sharper signals when NaCl was added, which partially compensated the sensitivity drop due to increased conductivity. Still for more aggregation-prone protein like REF2-I the overall sensitivity achieved with the use of sole Arg+Glu is better or at least comparable with the sensitivity achieved when Arg+Glu is combined with NaCl. Even if the overall sensitivity is the same, the ability to use less material and shorter pulses will present significant advantages. For less "stubborn" proteins, compared at constant concentrations, the expected gain from the sole usage of Arg+Glu should be the same as for SF2, i.e., up to 2-fold. Therefore, the use of arginine glutamate as a sole solubilizing salt (i.e., without NaCl) is justified, even if the maximum achievable protein concentration might be somewhat lower than when NaCl is also present.

The increase in experimental sensitivity and shorter pulse length observed in samples solubilized by sole Arg+Glu can be explained by the low conductivity of this salt (Table 1), which in turn can be explained by the low mobility of its amino acid ions. The large size, presence of zwitter-ionic groups and ability to form multiple hydrogen bonds should all contribute to low mobility of both cations and anions of this salt. As the protein solubility limit is proportional to the amounts of Arg+Glu added [10,11], the increased concentration of these amino acids above 50 mM may be tried to achieve the required protein concentration level in some cases (Hautbergue, Golovanov, unpublished observations). This can be done without a substantial penalty of increasing conductivity. The 50 mM Arg+Glu has a pH buffering capacity, although weak, due to the presence of total six titratable charged groups per anion-cation pair. To increase pH stability further if required, small quantities of low-conductivity buffering salts identified previously [4] can be added.

The obvious concern, associated with using a relatively high concentration of proton-containing additives in the NMR sample solution, is that the strong signals from these protons will mask useful protein signals and increase t_1 noise in multi-dimensional experiments. Previously it was noted [10] that using 50 mM non-deuterated Arg+Glu does not obscure the fingerprint amide region of the proton

Table 1
The influence of solubilizing salts on Dc conductivity σ, proton 90° pulse length and overall sensitivity of NMR experiments using cryoprobe

Salts added to sample	σ, mS/cm	Pulse[a]	Sensitivity[b]	Relative concentration[c]
50 mM Arg+Glu	1.87	1.09/1.05	1/1	1/1
50 mM Arg+Glu + 100 mM NaCl	11.1	1.52/1.39	0.68/0.5	1/1
100 mM NaCl[d]	11.7[d]	1.52/1.44	0.17/0.2	0.22/0.27

[a] The proton 90° pulse length is shown relative to that measured for typical protein sample in de-ionized water.
[b] The relative experimental sensitivity is shown.
[c] The protein concentrations in samples are shown relative to the maximum concentrations achieved with 50 mM Arg+Glu alone.
[d] Additionally 20 mM sodium phosphate buffer was added to stabilize sample pH; the conductivity without this buffer was 10.16 mS/cm. The data for REF2-I and SF2 is shown as (REF/SF2).

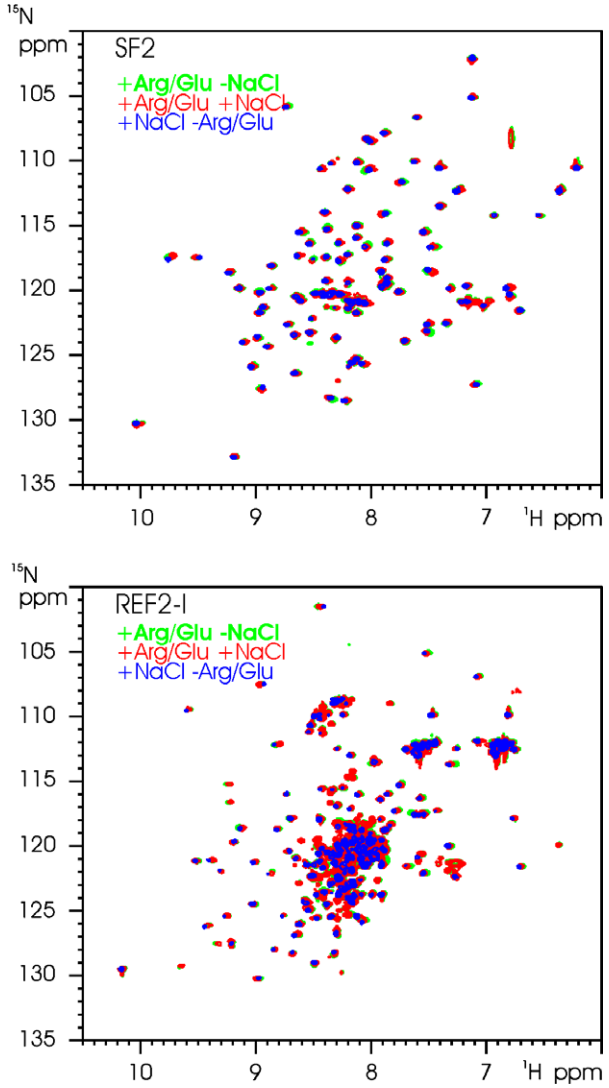

Fig. 2. Overlayed ^{1}H-^{15}N HSQC spectra of ^{15}N-labelled proteins SF2 (top panel) and REF2-I (bottom panel) concentrated and acquired in different solutions. Green: samples concentrated to the solubility limit in 50 mM L-Arg, 50 mM L-Glu. Red: as green but with dry NaCl added to the same samples to the final 100 mM concentration. Blue: protein samples concentrated in 100 mM NaCl, 20 mM phosphate buffer.

spectra, because amide signals of free amino acids are broadened by exchange beyond detection. Although the broad signals from arginine guanidinium group protons start to appear at lower pH (<6), they are situated away from the "interesting" proton amide region (Golovanov, unpublished observations). In homonuclear ^{1}H experiments the signals originating from the Arg+Glu sidechains in the aliphatic part of the spectra do interfere with the protein signals [10]. Therefore, non-deuterated L-Arg and L-Glu are not suitable for such homonuclear experiments. The situation is completely different if using ^{15}N or ^{13}C,^{15}N isotopically-labeled protein samples. In this case we found that the signals from non-labeled 50 mM Arg+Glu are automatically filtered out by all routine heteronuclear NMR experiments used for protein sequence-specific assignment and structure calculation. In ^{13}C-edited NOESY spectra, the natural abundance signals from Arg+Glu may give rise to t_1 noise strips, similar to those sometimes arising from the flexible moieties of the protein (Golovanov, unpublished observations). However, from our experience and others [10,12,14–17], no additional measures are needed to suppress signals from $\leqslant$50 mM Arg+Glu when using isotopically-labeled proteins, and no deuteration of these amino acids is required, making this method cost-effective for routine studies.

This inexpensive salt, arginine glutamate, should be useful both for "solubility limited" and "quantity limited" proteins as it allows improvement of the filling factor for the probehead and placement of the sample closer to the RF coils, without introducing significant ring currents in the potential "sample RF hot spots" [6], thus avoiding sensitivity losses. It can be used with the normal 5 mm or Shigemi sample tubes and with the older generations of "non-salt-tolerant" cryoprobes. With conductivity-related losses reduced, maximizing the sample volume should lead to maximal sensitivity. For quantity-limited proteins (i.e., when comparing the samples with the same protein concentration) up to 2-fold gain in sensitivity can be achieved. Slightly higher viscosity of arginine glutamate solution may reduce this gain, but this increase in viscosity may be in turn compensated by the small raise in temperature. For solubility-limited proteins the gain can be much higher: here a 5- to 6-fold increase in overall experimental sensitivity was obtained compared with the typical "traditional" sample buffer. The method is therefore most beneficial for the challenging proteins which are affected by aggregation. (Proteins which are not affected by aggregation likely can be run in de-ionized water instead). The practice shows that the individual aggregation-related behavior varies

from protein to protein, making "optimal" sample preparation protocols difficult to generalize. The current study however demonstrates the possibility to avoid using highly-conductive salt altogether while improving protein solubility significantly. We are anticipating that the low-conductivity arginine glutamate salt might routinely replace the traditional high-conductivity solubilizing salts for isotopically-labeled protein NMR studies and partially resolve the long-standing issue with the loss of sensitivity of the cryoprobe equipment for salty samples.

3. Experimental

The proteins were obtained as described previously [15,17]. The initial buffer content of purified protein solutions (with protein concentration <1 mg/ml) was then exchanged. All NaCl and phosphate buffer from the samples were removed using a Hi-Trap desalting column, exchanging the "traditional" sample buffer for a solution containing only 50 mM L-Arg, 50 mM L-Glu, 5 mM β-mercaptoethanol and 1 mM DTT, pH 6.4. The proteins were then concentrated up to their solubility limit in an Amicon stirred ultrafiltration cell. In the separate experiment, the protein samples were diluted below 0.5–1.0 mg/ml in a solution containing 100 mM NaCl, 20 mM phosphate, 5 mM β-mercaptoethanol and 1 mM DTT, pH 6.4. The buffer was then exchanged by a series of three dilution-concentration cycles by ultrafiltration, using an Amicon stirred cell. Protein concentration was measured using Bradford reagent. Milli-Q water was used for all solution preparations. The amino acids L-Arg and L-Glu were purchased from Sigma. It is important not to use these amino acids in the form of salts: e.g. L-Arg·HCl or L-Glu·Na are not suitable as they would introduce additional highly-conducting high-mobility ions into the solution. The buffer conductivity was measured by AKTA (GE Healthcare) on-line conductivity module of the UV-900 monitor. The NMR experiments were conducted on a Bruker 600 MHz Avance DRX spectrometer equipped with 5 mm TXI Cryoprobe, using the standard 5 mm sample tubes (535-pp, Wilmad Co.). All the spectra used for the comparison were collected in the identical conditions, with the same number of scans. Spectra of REF2-I and SF2 were run at 35 °C and 20 °C, respectively. The HSQC spectra were acquired with 1024 and 128 complex points in the direct and indirect dimensions, respectively.

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