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ARTICLE

Glutarate and *N*-acetyl-L-glutamate buffers for cell-free synthesis of selectively ^{15}N-labelled proteins

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Abstract Cell-free protein synthesis provides rapid and economical access to selectively ^{15}N-labelled proteins, greatly facilitating the assignment of ^{15}N-HSQC spectra. While the best yields are usually obtained with buffers containing high concentrations of potassium L-glutamate, preparation of selectively ^{15}N-Glu labelled samples requires non-standard conditions. Among many compounds tested to replace the L-Glu buffer, potassium *N*-acetyl-L-glutamate and potassium glutarate were found to perform best, delivering high yields for all proteins tested, with preserved selectivity of ^{15}N-Glu labelling. Assessment of amino-transferase activity by combinatorial ^{15}N-labelling revealed that glutarate and *N*-acetyl-L-glutamate suppress the transfer of the ^{15}N-α-amino groups between amino acids less well than the conventional L-Glu buffer. On balance, the glutarate buffer appears most suitable for the preparation of samples containing ^{15}N-L-Glu while the conventional L-Glu buffer is advantageous for all other samples.

Keywords Cell-free protein synthesis · Combinatorial ^{15}N-labelling · ^{15}N-HSQC · Potassium glutarate · Potassium *N*-acetyl-L-glutamate · Selective ^{15}N-Glu labelling

Abbreviations

CI-BABP	Chicken *ileum* bile-acid binding protein
PpiB	*E. coli* peptidyl-prolyl *cis–trans* isomerase b
HSQC	Heteronuclear single quantum coherence
*h*CypA	Human cyclophilin A
WNVpro	West Nile virus NS2B/NS3 protease
DENpro	Dengue virus type 2 NS2B/NS3 protease

Introduction

Since the advent of a protocol for cell-free synthesis of proteins with high yields (Kigawa et al. 1999), cell-free protein synthesis has become an important tool in protein structural biology by NMR spectroscopy (Yokoyama et al. 2000; Vinarov et al. 2004; Etezady-Esfarjani et al. 2007). Cell-free protein synthesis saves costs by requiring only small amounts of isotope-labelled amino acids (Torizawa et al. 2004). It is uniquely suited for selective isotope labelling, as metabolic conversions of isotope-labelled amino acids are suppressed (Ozawa et al. 2004). The protein yields can be sufficient for recording 2D HSQC spectra straight of the reaction mixture, where isotope labelling allows selective observation of the protein synthesized even without protein purification (Guignard et al. 2002). Finally, proteins can be synthesized from linear DNA that has been amplified by PCR, enabling exceptional throughput rates (Kim and Hahn 2006; Wu et al. 2007; Jun et al. 2008; Seki et al. 2008).

In many projects, we have found cell-free protein synthesis most useful for assisting backbone resonance assignments by amino-acid type selective ^{15}N-labelling. This is achieved with the greatest efficiency in a combinatorial labelling scheme which allows identification of the amino-acid type of all ^{15}N-HSQC cross-peaks from a set of only five spectra (Wu et al. 2006). This labelling scheme is

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only practical with cell-free protein synthesis, as in vivo protein production in *E. coli* is invariably associated with severe isotope scrambling (McIntosh and Dahlquist 1990). The standard cell-free reaction protocol with an *E. coli* cell extract, however, employs a high concentration of potassium L-Glu (Kigawa et al. 1999; Apponyi et al. 2008), so that a different buffer must be used for economical preparation of samples containing ^{15}N-labelled Glu.

Use of much lower glutamate concentrations (e.g. 1.5 mM instead of 208 mM; Wu et al. 2006) almost always leads to substantially reduced yields. Replacing potassium L-Glu by potassium acetate or ammonium acetate has been proposed (Ozawa et al. 2004) but also results in somewhat lower protein yields (Matsuda et al. 2007). This led to the proposal to replace L-Glu by D-Glu which has been reported to produce similar yields without compromising the labelling efficiency of ^{15}N-Glu in the target protein (Matsuda et al. 2007). The success of this strategy demonstrates that glutamate racemase activity is well suppressed in the *E. coli* extract. In our hands, however, the potassium D-Glu buffer tends to produce lower protein yields than the original potassium L-Glu buffer. We therefore initiated a search for an alternative inexpensive buffer that could replace L-Glu in cell-free reactions with *E. coli* cell extracts. As the mechanism by which glutamate increases expression yields in the cell-free reactions is not understood, we took an empirical approach by testing a range of related compounds. In addition, we prepared combinatorially labelled samples to assess the isotope scrambling potential associated with the different buffers.

Materials and methods

Sample preparation

Cell-free protein synthesis

Proteins were synthesized in a cell-free *E. coli* coupled transcription-translation system, adopting a previously described protocol (Ozawa et al. 2005a; Apponyi et al. 2008). Specifically, S30 extract was made at 30 or 37°C from the Rosetta/pRARE strain. The plasmid pKO1166 was employed to produce T7 RNA polymerase in situ during the cell-free reaction (Ozawa et al. 2005a). As the T7 RNA polymerase is rich in alanine, the concentration of Ala provided in the reaction was increased to 2 mM. All proteins were expressed from plasmid DNA in a dialysis system at pH 7.5, containing 0.5 ml of reaction mixture in 5 ml of outside buffer or 0.2 ml of reaction mixture in 2 ml of outside buffer. S30 extract made at 30°C was used for all reactions performed at 30 or 34.5°C.

Isotope scrambling associated with different buffers was studied by preparing combinatorially ^{15}N-labelled samples of *E. coli* PpiB, using plasmid pKO1154 (Ozawa et al. 2005b) which harbours the gene in the T7-promoter vector pETMCSI (Love et al. 1996). Five samples were prepared for combinatorial labelling (Wu et al. 2006; Ozawa et al. 2006), containing ^{15}N-labelled Leu, Arg, Asp, Asn, Tyr, His and Cys in sample 1, ^{15}N-labelled Ala, Lys, Arg, Phe, Gln, Met, Cys and Trp in sample 2, ^{15}N-labelled Gly, Ile, Lys, Thr, Asn, His and Trp in sample 3, ^{15}N-labelled Ser, Val, Ile, Gln, Tyr, Met, His and Trp in sample 4 and ^{15}N-labelled Glu, Val, Thr, Asp, Phe, Met and Cys in sample 5. All five samples were prepared in 100 mM potassium *N*-acetyl-L-Glu buffer or 100 mM potassium glutarate buffer. In addition, samples 1–4 were prepared in 208 mM L-Glu buffer as usual. Cell-free synthesis of PpiB was carried out for about 7 h at 34.5°C.

NMR analysis

Samples were transferred into NMR buffer (50 mM sodium phosphate, 1 mM DTT, pH 6.2 for *E. coli* PpiB; Kariya et al. 2000) by dialysis. Before NMR measurements, D_2O was added to 10% (v/v). ^{15}N-HSQC spectra were recorded at 28°C on Bruker AV600 and AV800 NMR spectrometers, using $t_{1max} = 36$ ms and $t_{2max} = 131$ ms. The cross-peaks in the ^{15}N-HSQC spectra of PpiB were assigned using the published assignments (BMRB accession code 4765; Kariya et al. 2000).

Results

Protein yields

The compounds tested as buffers to substitute glutamate were selected for general structural similarity with glutamate and commercial availability. Table 1 presents an overview of the compounds tested and the results obtained. Only a subset of the compounds delivered acceptable protein yields with PpiB, a protein that is particularly easy to produce in high yield (about 2 mg of protein per ml of reaction mixture) under different conditions (Kariya et al. 2000; Guignard et al. 2002).

Initial results showed that 104 mM potassium glutarate gave expression yields close to those obtained with 208 mM L-Glu. In order to understand the buffer requirements in more detail, we explored a range of additional buffers. Iminodiacetic acid proved to be unsuitable and γ-aminobutyrate turned out to be inhibitory even in the presence of glutarate. This indicates that dicarboxylic acids have special potential to improve protein yields in cell-free reactions.

100 mM succinate and adipate were found to act as acceptable substitutes for L-Glu, although the yields tended to be somewhat lower than for L-Glu, with significantly greater variability between different proteins. Malonate,

Table 1 Chemical compounds tested as substitutes for L-Glu[a]

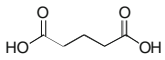
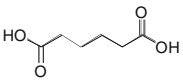
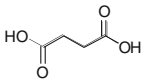
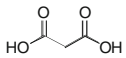
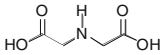
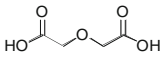
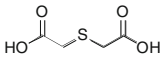
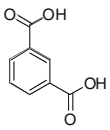
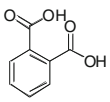
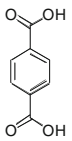
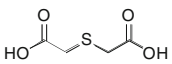
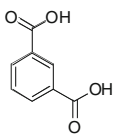
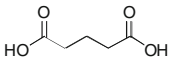
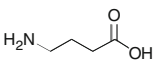
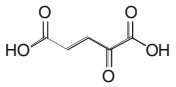
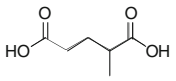
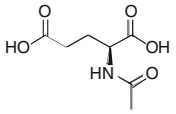
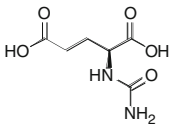
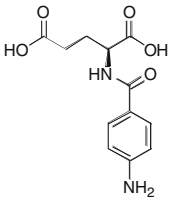
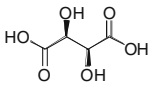
Name	Chemical Structure	Assayed Concentration (mM)	Expression Level[b]
glutaric acid		*100*, 208	+ (PpiB) ++ (ArgN, DnaI-N, RRM1, *h*CypA)
adipic acid		50, *100*, 200	++ (PpiB)
succinic acid		50, *100*, 200	++ (PpiB, RRM1, τ_C22) + (τ_C16, ArgN, χ, ε, ubiquitin)
malonic acid		20, 40, 60, 80, 100, 120, 140, 160, 180, 208	- (PpiB)
iminodiacetic acid		25, 50, 100, 200	- (PpiB)
diglycolic acid		45, 112.5, 157.5, 225	- (PpiB)
thiodiglycolic acid		20, 50, 70, *100*, 208	+ (PpiB) - (RRM1, ArgN, cI-BABP)
isophthalic acid		20, 50, 70, 100	- (PpiB)
phthalic acid		20, 50, 70, 100	- (PpiB)
terephthalic acid		20, 50, 70, 100	- (PpiB)
thiodiglycolic acid + isophthalic acid	 + 	70 + 20	- (PpiB)
glutaric acid + γ-aminobutyric acid	 + 	104 + 104	- (PpiB)

Table 1 continued

α-ketoglutaric acid		50, *70*, 100, 150	- (*h*CypA)[c]
2-methylglutaric acid		50, 70, *100*, 150	- (*h*CypA)[c]
N-acetyl-L-glutamic acid		50, 70, *100*, 150, 175, 208	++ (*h*CypA, RRM1, PpiB, WNVpro, DENpro)[c]
N-carbamyl-L-glutamic acid		100	++ (*h*CypA)[c]
N-(4-aminobenzoyl)-L-glutamic acid		50, 70, 100	- (*h*CypA)[c]
D-(-)-tartaric acid		2, 5, 10, 12, 50, 100, 200	- (PpiB)

[a] Compounds were titrated with potassium hydroxide to pH 7.5 before use in the cell-free reaction buffer. The concentrations indicated are the final concentrations in the cell-free reaction mixture. The cell-free reactions were carried out at 37°C using S30 extract made at 37°C unless indicated otherwise. Different temperatures for the S30 preparations and cell-free reactions had a much smaller impact on protein yields than the buffer. *Minus* and *plus* signs indicate that the protein in the new buffer was, respectively, less or more than half of the yield in 208 mM potassium L-Glu buffer. A ++ symbol indicates protein yields comparable to those achieved in 208 mM L-Glu buffer. Buffer concentrations for optimal yields are highlighted in italics

[b] Assays were carried out with the proteins indicated in brackets. PpiB: *E. coli* peptidyl-prolyl *cis-trans* isomerase; *h*CypA: human cyclophilin A; RRM1: hnRNPLL RRM1 domain (Wu et al. 2008); ArgN: *N*-terminal domain of the *E. coli* arginine repressor (Sunnerhagen et al. 1997); DnaI-N: *N*-terminal domain of *B. subtilis* DnaI (Loscha et al. 2009); WNVpro: West Nile virus NS2B/NS3 protease (Erbel et al. 2006); τ_C16, τ_C22, χ (Ozawa et al. 2005a) and ε (Ozawa et al. 2008): subunits of the *E. coli* DNA polymerase III (Su et al. 2007); cI-BABP: chicken ileum bile-acid binding protein (Guariento et al., 2008); DENpro: dengue virus type 2 NS2B/NS3 protease (Erbel et al. 2006)

[c] Assays performed at 30 or 34.5°C using S30 extract prepared at 30°C

however, gave poor results at any concentration, showing that the spacing between the carboxyl groups is important. Nonetheless, neither isophthalic acid and the related isomers nor tartaric acid worked. This shows that the range of suitable dicarboxylates is limited. Despite its close structural similarity to glutaric acid, however, diglycolic acid gave low expression yields whereas thiodiglycolic acid was better. Nonetheless, the performance of thiodiglycolic acid was protein specific, giving good yields for PpiB but not for a range of other proteins.

We subsequently tested L-Glu derivatives that are less prone to metabolic conversions by the cell-free extract than free L-Glu. Three L-Glu compounds were tested, where the amino group was protected as an amide with a carbamoyl, acetyl or 4-aminobenzoyl group, respectively. Whereas, the 4-aminobenzoyl derivative led to poor expression yields, the carbamoyl and acetyl derivatives produced good yields.

In view of the much lower cost of the acetyl derivative, we analysed the performance of the *N*-acetyl-L-Glu buffer in more detail together with that of the glutarate buffer. The optimal concentration of potassium *N*-acetyl-L-Glu was found to be about 100 mM, as illustrated in Fig. 1a for human cyclophilin A (*h*CypA). The optimal concentration of potassium glutarate was equally in the 100–120 mM range (Fig. 1b). Under these conditions, the yields obtained for 5 different proteins [PpiB, *h*CypA, West Nile virus NS2B/NS3 protease, dengue virus NS2B/NS3 protease (Erbel et al. 2006), hnRNPLL RRM1 domain (Wu et al. 2008)] with *N*-acetyl-L-Glu were

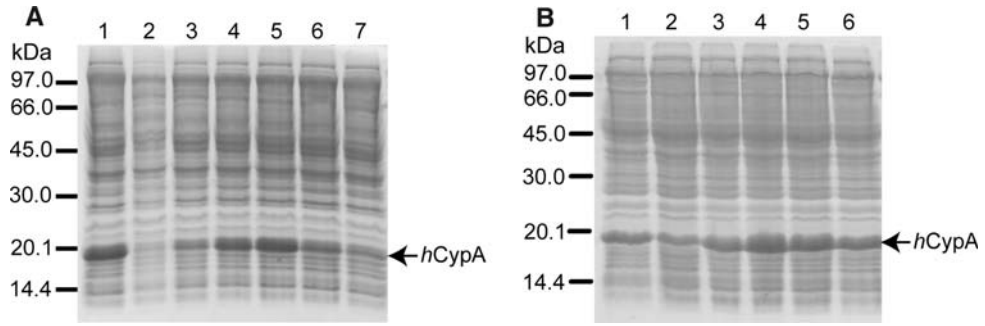


Fig. 1 Performance of the potassium *N*-acetyl-L-Glu buffer in cell-free protein synthesis. The complete reaction mixture was loaded on SDS/PAGE (15%, stained with Coomassie Blue). **a** Optimisation of the *N*-acetyl-L-Glu concentration for cell-free synthesis of *h*CypA (6.5 hours reaction time at 34.5°C). *Lane 1*: reference sample expressed in 208 mM potassium L-Glu. *Lanes 2–7*: expression in decreasing concentrations of potassium *N*-acetyl-L-Glu (208 mM in lane 2, 175 mM in lane 3, 150 mM in lane 4, 100 mM in lane 5, 70 mM in lane 6, 50 mM in lane 7). **b** Optimisation of the potassium glutarate concentration for cell-free synthesis of *h*CypA (14 h reaction time at 30°C). *Lane 1*: reference sample expressed in 208 mM potassium L-Glu. *Lanes 2–6*: expression in decreasing concentrations of potassium glutarate (lane 2: 208 mM, lane 3: 150 mM, lane 4: 120 mM, 100 mM in lane 5, 80 mM in lane 6)

comparable to those obtained with 208 mM potassium L-Glu (Fig. S1). 80 mM glutarate produced in most cases slightly reduced yields compared to 100 mM *N*-acetyl-L-Glu (Fig. S2). While a higher concentration of glutarate improved the yield of *h*CypA (Fig. 1b) this was found to inhibit the production of PpiB (data not shown). In almost all cases, the yields obtained with 208 mM L-Glu were better than those obtained with 250 mM D-Glu (Fig. S2) and maximal yields were obtained after about 6 h. Extension of the reaction time to 15 h had little effect (Figs. S1, S2). In summary, the *N*-acetyl-L-Glu and glutarate buffers can serve as substitutes for L-Glu, but the glutarate buffer may be more sensitive to the protein identity.

In order to make the buffer less sensitive to protein identity, we also investigated a buffer made of 50 mM potassium *N*-acetyl-L-Glu and 50 mM potassium glutarate. Initial results were encouraging, showing protein yields comparable to those obtained with 208 mM L-Glu for all proteins tested (Fig. S4).

Combinatorial ^{15}N-labelling

The performance of the *N*-acetyl-L-Glu and glutarate buffers for the production of proteins with selectively ^{15}N-labelled amino acids was explored by preparing five samples of PpiB with combinatorial labelling (Wu et al. 2006). For comparison, four of the samples (samples 1–4 that do not contain ^{15}N-Glu) were, in addition, produced using the conventional potassium L-Glu buffer. The great majority of amino acids yielded cross-peaks only in those samples, where they had been provided as ^{15}N-labelled amino acids. Asn, Asp, Gln, Glu, Gly, Trp, and Tyr, however, showed signs of amino-transferase activity as manifested by the appearance of ^{15}N-HSQC cross-peaks of supposedly unlabelled residues (chemical cross-talk). As expected for enzymatic conversions prior to protein synthesis, different residues of the same amino-acid type displayed the same level of chemical cross-talk.

For example, Fig. 2a shows that ^{15}N-Gly cross-peaks could be observed also in sample 4, although that sample was produced without ^{15}N-Gly. This cross-talk can be explained by metabolic conversion from ^{15}N-Ser present in sample 4. Similarly, unwarranted cross-peaks of Gln and Asn were observed for sample 5 (Fig. 2b, c), presumably by conversion from ^{15}N-Glu. Unwarranted cross-peaks of Tyr and Trp also appeared in sample 5 when prepared with *N*-acetyl-L-Glu buffer, but were strongly suppressed in the corresponding sample prepared with glutarate (Fig. 2d, g).

The unwarranted cross-peaks were always weaker than the desired cross-peaks, except in the cases of Gln and Asn (Fig. 2b, c). Gln cross-peaks were particularly affected in samples prepared with L-Glu buffer (Fig. 2b, left panel). The labelling pattern experimentally observed for Gln would lead to confusion with Met, as the labelling scheme was designed to show cross-peaks of Met in samples 2, 4, and 5. In the case of Asn, the undesired peak in sample 5 was more intense than the desired peak in sample 3, but as no other amino acid was ^{15}N-labelled in samples 1, 3 and 5, the labelling pattern remains unambiguous. Similarly, cross-talk did not compromise the uniqueness of the labelling patterns of Tyr, Asp, Glu and Trp.

For samples 1–4, the L-Glu buffer suppressed cross-talk better than the other buffers, indicating that high concentrations of L-Glu are inhibitory for some of the metabolic conversions. Therefore, if reduced cross-peak intensities of Gln are acceptable, L-Glu is the best buffer for samples 1–4. Among the proteins produced with combinatorial labelling in our laboratory, the data of Fig. 2 present the strongest cross-talk effects.

In order to assess the performance of the buffer made of 50 mM potassium *N*-acetyl-L-Glu and 50 mM potassium

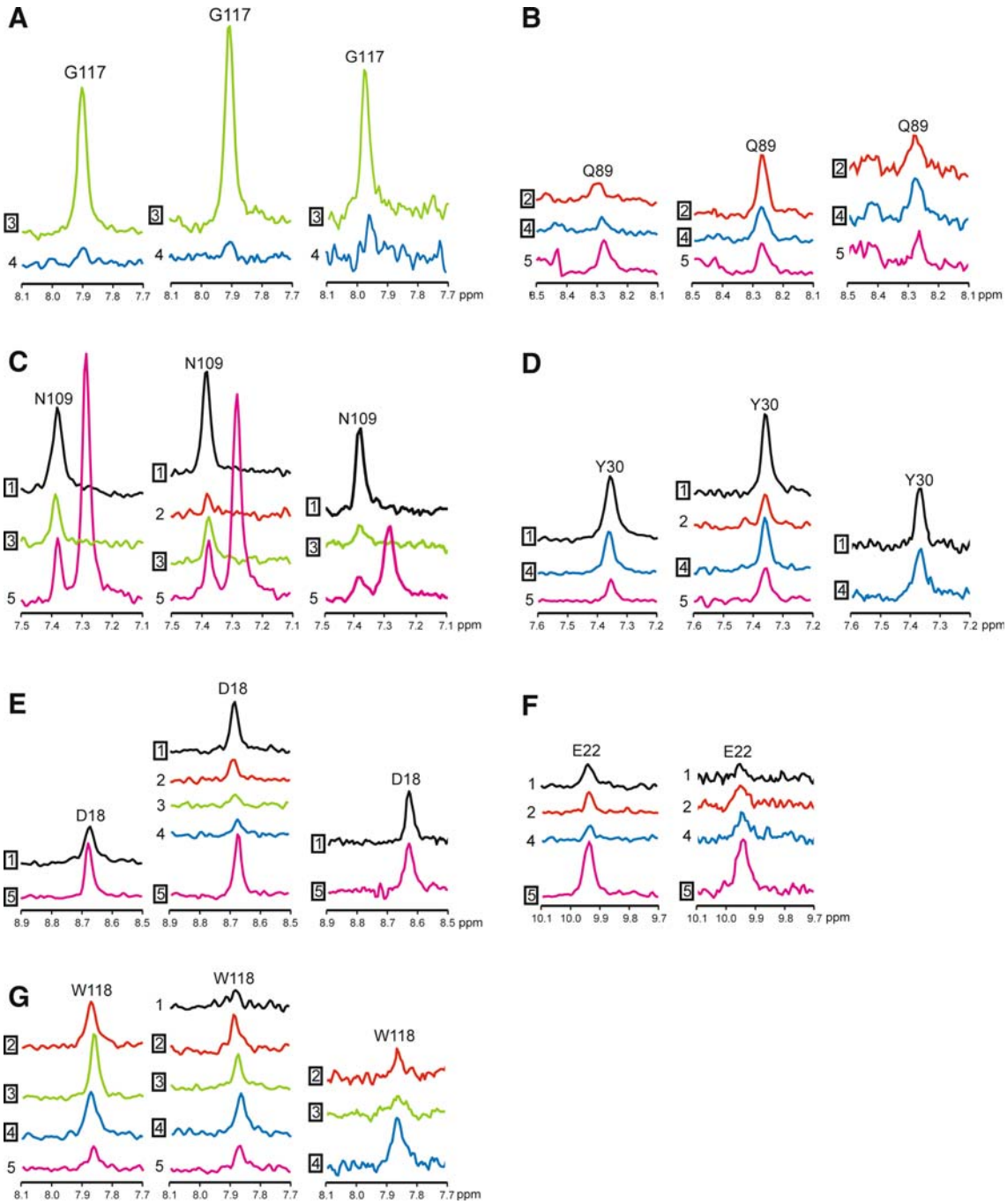


Fig. 2 Comparison of amino-transferase activity in cell-free protein synthesis using L-Glu, *N*-acetyl-L-Glu and glutarate buffers. Cross-sections were taken through ^{15}N-HSQC cross-peaks of selected residues of PpiB produced with combinatorial isotope labelling. Each subfigure shows three panels of cross-sections, where samples 1–5 were prepared with different buffers. In the *left panel*, samples 1–4 were prepared in L-Glu buffer and sample 5 in *N*-acetyl-L-Glu buffer. In the *central panel*, samples 1–5 were prepared in *N*-acetyl-L-Glu buffer. In the *right panel*, samples 1–5 were prepared in glutarate buffer. (Sample 5 was prepared only once with *N*-acetyl-L-Glu, but dialyzed against the NMR buffers of samples 1–4 produced with L-Glu or *N*-acetyl-L-Glu, respectively, for maximal reproducibility of the cross-peak positions.) Within each panel, the cross-sections were plotted with the same scaling factor. The cross-sections are labelled with the sample numbers. *Boxes* identify sample numbers where the respective amino acid was added to the cell-free mixture in ^{15}N-labelled form and a ^{15}N-cross-peak is expected. Cross-sections were plotted only if a cross-peak could be observed. The cross-peaks of **a** G117, **b** Q89, **c** N109, **d** Y30, **e** D18, **f** E22, and **g** W118 are shown. The ^{15}N-labelled amino acids used for the preparation of samples 1–5 were, respectively: Leu, Arg, Asp, Asn, Tyr, His, Cys (sample 1); Ala, Lys, Arg, Phe, Gln, Met, Cys, Trp (sample 2); Gly, Ile, Lys, Thr, Asn, His, Trp (sample 3); Ser, Val, Ile, Gln, Tyr, Met, His, Trp (sample 4); Glu, Val, Thr, Asp, Phe, Met, Cys (sample 5). Consequently, ^{15}N-Gly was present at the start of the reaction only in sample 3, ^{15}N-Gln only in samples 2 and 4, ^{15}N-Asn only in samples 1 and 3, ^{15}N-Tyr only in samples 1 and 4, ^{15}N-Asp only in samples 1 and 5, ^{15}N-Glu only in sample 5, and ^{15}N-Trp only in samples 2, 3 and 4

glutarate, we also prepared sample 5 in this buffer mixture. The NMR spectrum showed cross-talk for Tyr and Trp at a level between those observed in the samples prepared in 100 mM *N*-acetyl-L-glutamate or 100 mM glutarate.

Discussion

Glutamate plays a key role in *E. coli* metabolism. In addition, it serves as an important intracellular osmolyte, with concentrations ranging from 30 to over 250 mM, depending on the osmolarity of the growth medium (Richey et al. 1987). It is thus no surprise that *E. coli* cell-free extracts can tolerate high concentrations of glutamate.

Similarly, the intracellular potassium concentration in *E. coli* is high (about 300 mM), varying about two-fold depending on extracellular ion concentrations (Kuhn and Kellenberger 1985). In contrast, the intracellular sodium concentration is almost 100-fold smaller (Castle et al. 1986). Potassium has also been shown to be essential for cell-free protein synthesis (Kigawa et al. 1995). Therefore, we only investigated potassium salts for their potential to substitute potassium glutamate.

Mg^{2+} is another cation present at high concentration in *E. coli*. Typical intracellular concentrations are about 100 mM, but only about 1–5 mM of the Mg^{2+} is free (Alatossava et al. 1985). While the concentration of Mg^{2+} is an important parameter in cell-free protein synthesis, best expression yields are obtained with total Mg^{2+} concentrations of about 20 mM (Apponyi et al. 2008). Glutamate is a weak chelator of Mg^{2+} ions with a dissociation constant of about 10 mM (at pH 7 and 20°C; Sillen and Martell 1964) and this buffering activity has been identified as an important parameter for the ATPase activity of the termination factor rho (Zou and Richardson 1991). Notably, however, rho activity is not essential for cell-free protein synthesis. Quite generally, the Mg^{2+} chelating activity of glutamate seems to be of little importance for the overall protein yield in cell-free protein synthesis, as the optimum Mg^{2+} concentration varied only little between the different dicarboxylate buffers of Table 1 that produced maximal protein yields at much lower buffer concentrations than glutamate (see also Fig. S3).

The present results show that 100 mM potassium *N*-acetyl-L-Glu or potassium glutarate can replace 208 mM potassium L-Glu as a buffer in cell-free protein synthesis based on an *E. coli* S30 cell extract. Both buffers consistently lead to protein yields comparable with those obtained with L-Glu and are suitable for overnight reactions. Both allow the preparation of selectively ^{15}N-Glu labelled samples, although *N*-acetyl-L-Glu is involved in *E. coli* metabolism (Fernández-Murga and Rubio 2008). Amino-transferase activity, however, is better suppressed by the L-Glu buffer which can suppress the transfer of the ^{15}N-label by product inhibition of some of the metabolic enzymes or by dilution of the label in metabolic pathways involving L-Glu. Therefore, we recommend the L-Glu buffer for the preparation of ^{15}N-labelled samples not containing ^{15}N-Glu and either *N*-acetyl-L-Glu or glutarate or mixtures of *N*-acetyl-L-Glu and glutarate for ^{15}N-Glu labelled samples.

Transfer of ^{15}N-labelled α-amino groups between different amino acids can be sufficiently efficient for some amino acids to confuse the results from combinatorial ^{15}N-labelling experiments even when the proteins are produced by cell-free synthesis. Nonetheless, although unwarranted ^{15}N-HSQC cross-peaks were observed for Asn, Asp, Gln, Glu, Gly, Trp, and Tyr, these were almost always weaker than the expected cross-peaks. In addition, identification of the 19 non-proline amino acids remains unambiguous provided that the new labelling pattern created by the cross-talk between different samples remains unique. This can readily be achieved, as five samples can encode $2^5 = 32$ different patterns. In practice, the labelling scheme of Wu et al. (2006) always yielded unambiguous assignments, except that Gln could be confused with Met. This problem could be solved by labelling Gln (rather than Met) in samples 2, 4 and 5, and Met (rather than Gln) in samples 2 and 4. In this case, Glu and Gln would both be ^{15}N-labelled in sample 5, so that the conversion of Glu to Gln would have no net effect. As Met is inert towards amino-transferase reactions, this will create distinct labelling patterns for Met and Gln.

In our experience, different proteins prepared with combinatorial ^{15}N-labelling can produce different levels of cross-talk. This effect may arise from different protein production rates, as metabolically converted amino acids accumulate during the course of the cell-free reaction.

Besides *N*-acetyl-L-Glu and glutarate, succinate was found to be an inexpensive buffer suitable for ^{15}N-Glu labelling. Protein yields achieved with succinate, however, are typically only half of those obtained with *N*-acetyl-L-Glu or glutarate, although, in the case of the hnRNPLL RRM1 domain (Wu et al. 2008), very similar yields were obtained with succinate and glutamate.

It is remarkable that high yields in cell-free protein synthesis depend so critically on dicarboxylic acids and, specifically, on glutamate or related molecules such as *N*-acetyl-L-Glu, *N*-carbamyl-L-Glu or glutarate. It is also interesting that all these compounds produced optimal protein yields at about half the buffer concentration (about 100 mM) of L-Glu (about 200 mM) that carries a smaller net charge. Considering that the performance of the buffers sometimes depends on the protein, one might speculate that the buffers assist the folding, or extension and release of the polypeptide chain from the ribosome. Since a buffer

containing 50 mM L-Glu and L-Arg in 1:1 ratio has been shown to increase the solubility and long-term stability of proteins (Golovanov et al. 2004), we investigated the effect of 100 mM L-Arg in a reaction mixture containing 208 mM L-Glu for the expression of *h*CypA. The presence of L-Arg proved inhibitory (data not shown). The molecular mechanism underpinning the need for glutamate or glutamate-related compounds in cell-free protein synthesis thus remains unclear.

Concluding remarks

The present study explored alternative buffer systems for cell-free protein synthesis using an *E. coli* S30 cell extract. Potassium *N*-acetyl-L-Glu and potassium glutarate emerged as alternative buffers capable of producing protein yields equivalent to those achieved with potassium L-Glu. *N*-acetyl-L-Glu and glutarate are suitable for the preparation of selectively ^{15}N-Glu labelled as well as uniformly ^{15}N-labelled samples. 1:1 mixtures of *N*-acetyl-L-Glu and glutarate gave equally good protein yields and may be less sensitive to the protein identity. Neither of the alternative buffers consistently enhanced the protein yields over those obtained with the L-Glu buffer, suggesting that protein yields are limited by other parameters. Identification of these parameters is the subject of ongoing research.

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