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ARTICLE

Suppression of isotope scrambling in cell-free protein synthesis by broadband inhibition of PLP enymes for selective ^{15}N-labelling and production of perdeuterated proteins in H_2O

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Abstract Selectively isotope labelled protein samples can be prepared in vivo or in vitro from selectively labelled amino acids but, in many cases, metabolic conversions between different amino acids result in isotope scrambling. The best results are obtained by cell-free protein synthesis, where metabolic enzymes are generally less active, but isotope scrambling can never be suppressed completely. We show that reduction of *E. coli* S30 extracts with $NaBH_4$ presents a simple and inexpensive way to achieve cleaner selective isotope labelling in cell-free protein synthesis reactions. The purpose of the $NaBH_4$ is to inactivate all pyridoxal-phosphate (PLP) dependent enzymes by irreversible reduction of the Schiff bases formed between PLP and lysine side chains of the enzymes or amino groups of free amino acids. The reduced S30 extracts retain their activity of protein synthesis, can be stored as well as conventional S30 extracts and effectively suppress conversions between different amino acids. In addition, inactivation of PLP-dependent enzymes greatly stabilizes hydrogens bound to α-carbons against exchange with water, minimizing the loss of α-deuterons during cell-free production of proteins from perdeuterated amino acids in H_2O solution. This allows the production of highly perdeuterated proteins that contain protons at all exchangeable positions, without having to back-exchange labile deuterons for protons as required for proteins that have been synthesized in D_2O.

Keywords Cell-free protein synthesis · Isotope scrambling · $NaBH_4$ · Pyridoxal phosphate · Selective ^{15}N labelling

Introduction

Selective labelling of proteins with isotope labelled amino acids is essential for NMR studies of biomolecular systems of high molecular weight and investigations of active sites of proteins, both for improving spectral resolution and as a tool to assist resonance assignments. Unfortunately, in vivo protein expression often leads to scrambling of the isotope labels between different amino acids, preventing especially the production of selectively ^{15}N- and ^{2}H-labelled samples (LeMaster 1990; McIntosh and Dahlquist 1990; Shortle 1994; Takeuchi et al. 2007). These problems can only partially be overcome by using transaminase-deficient and auxotrophic *E. coli* mutants for amino-acid selective labelling of proteins in vivo (LeMaster 1990; McIntosh and Dahlquist 1990; Muchmore et al. 1989; Waugh 1996). Much cleaner NMR spectra can be obtained by cell-free protein synthesis, as the amino acid metabolism is compromised under in vitro conditions (Kigawa et al. 1995; Kainosho and Güntert 2010; Ozawa et al. 2004, 2006; Shi et al. 2004; Staunton et al. 2006; Sobhanifar et al. 2010; Yabuki et al. 1998). Nonetheless, some undesired enzymatic activities persist even in cell-free extracts. In particular, the α-amino groups of the amino acids Glu, Asp, Gly and Ser are prone to transfer to other amino acids, as these amino acids are central to the amino acid metabolism.

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In addition, it has been shown that cell-free extracts catalyse ^{2}H–^{1}H exchange at the α- and β-positions of ^{2}H-labelled amino acids (Etezady-Esfarjani et al. 2007). This is unfortunate, as cell-free synthesis of proteins from $^{2}H/^{15}N/^{13}C$-labelled amino acids in H_2O medium could deliver perdeuterated proteins with ^{1}H bound to all nitrogen and oxygen atoms which would be an ideal isotope labelling pattern for many NMR experiments. Conventionally, this labelling pattern is generated by protein production in D_2O medium followed by ^{2}H–^{1}H exchange of labile protons, but the conditions required for ^{2}H–^{1}H exchange of solvent inaccessible amides are often too harsh for proteins susceptible to irreversible denaturation or degradation.

It has been shown that some of the isotope scrambling in *E. coli* cell extracts can be partially suppressed by the use of high concentrations of ammonium acetate (Ozawa et al. 2004), dicarboxylic acids (Jia et al. 2009), or inhibitors of specific enzymes (Morita et al. 2004; Tong et al. 2008). No buffer condition, however, achieves complete suppression of all undesired metabolic pathways, while enzyme-specific inhibitors are expensive or unavailable for many of the *E. coli* enzymes. Here we show that a simple and inexpensive treatment of the S30 extract with $NaBH_4$ greatly suppresses a wide range of undesired metabolic side-reactions, including the exchange of C^{α} deuterons with water.

The purpose of the $NaBH_4$ treatment is to reduce the Schiff base (aldimine) formed between pyridoxal 5′-phosphate (PLP) and amino groups of either amino acid substrates or lysine residues of PLP-dependent enzymes, leading to irreversible inactivation. Selective reduction of the aldimine to an amine with $NaBH_4$ is an established method for the identification of residues forming Schiff bases with PLP in the active sites of transaminases (Fig. 1; Morino and Nagashima 1984).

PLP is a cofactor in all transaminases and many other enzymes involved in amino acid metabolism. Reduction of the Schiff base thus inhibits a wide range of PLP-catalyzed reactions, including transaminations, racemizations, decarboxylations, eliminations and replacements of electrophilic groups of amino acids (Berg et al. 2006; Eliot and Kirsch 2004). As PLP-dependent enzymes are not required for transcription and translation, their deactivation does not affect protein production.

Methods

S30 preparation

S30 extract was prepared at 30°C from *E. coli* BL21 (DE3) cells as described by Apponyi et al. (2008). Reduction with $NaBH_4$ was performed as follows. After thawing the S30 extract (1 mL) on ice overnight, it was diluted with 1 mL HEPES buffer (pH 7.6). 0.4 mL of a 100 mM solution of $NaBH_4$ in dry DMF was added step-wise into the mixture of S30 extract to a final nominal concentration of about 20 mM in a 10 mL Falcon tube, avoiding high local concentrations of $NaBH_4$ by gentle shaking. Hydrogen gas evolved during the reaction and the colour of the solution changed from yellow to pale yellow. After addition, the resulting mixture was incubated on ice for 10 min and then was dialysed three times against the cell-free reaction buffer.

Cell-free protein synthesis and purification of ^{15}N-labelled proteins

Proteins were synthesized by cell-free *E. coli* coupled transcription-translation, following an established protocol (Ozawa et al. 2004; Apponyi et al. 2008; Jia et al. 2009). A dialysis system with 0.5 mL inner reaction volume and 5 mL outside buffer was used and proteins were expressed from plasmid DNA at 30°C for 7 h. The target protein *E. coli* PpiB was transferred into NMR buffer (20 mM MES, pH 6.5, 2 mM DTT) using an Amicon Ultracel concentrator (Millipore, MWCO 3000) without further purification (Guignard et al. 2002). Before NMR measurements, D_2O was added to 10%.

Combinatorial labelling was performed as described (Wu et al. 2006; Jia et al. 2009), preparing five samples 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 Gln, Glu, Val, Thr, Asp, Phe and Cys in sample 5. All five samples were prepared in 208 mM potassium glutamate buffer except for sample 5 which was prepared in 100 mM N-acetyl-L-Glu buffer (Jia et al. 2009).

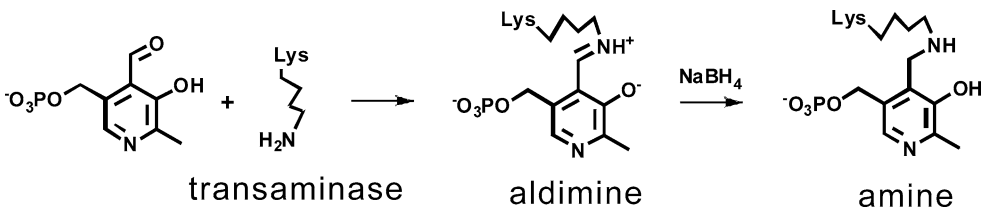

Fig. 1 Scheme of inhibition of PLP-dependent transaminases through reduction of the Schiff-base intermediate by $NaBH_4$

Cell-free synthesis of ubiquitin with perdeuterated amino acids

Samples of uniformly $^{2}H/^{15}N/^{13}C$-labelled human ubiquitin were prepared in cell-free reactions during 14 h at 30°C using conventional and reduced S30 extract in H_2O, using a reaction volume of 0.6 mL in 6 mL outside buffer. Instead of using the usual mixture of 20 amino acids, where each amino acid is present in 1 mM concentration, the protein was prepared from a commercially available mixture of $^{2}H/^{15}N/^{13}C$-labelled amino acids (Cambridge Isotope Laboratories, Andover MA, USA). 3.3 mL of a stock solution (0.1 g dissolved in 18 mL water) were used in each reaction. The pH was adjusted to 7.4 using KOH and the solution diluted to 3.5 mL with water prior to partitioning the amino acid mixture to achieve the same final concentration in the reaction mixture and outside buffer of the cell-free reaction. The ubiquitin construct used contained the T7 gene 10 N-terminal peptide MASMTG at the N-terminus and a His_6-tag at the C-terminus. The protein was purified using a Ni–NTA spin column (ProPur™ IMAC) as recommended by the manufacturer (Thermo Fisher Scientific), concentrated and the buffer exchanged to 20 mM HEPES, pH 6.5. The purity of the protein samples was verified by ^{15}N-HSQC spectra and the solvent exchanged to D_2O by repeated lyophilisation and dissolution in D_2O for measurements of ^{13}C-HSQC spectra. About 1.4 mg of protein were obtained in each reaction.

To assess isotope scrambling by enzymes in the cell-free extract under conditions of cell-free protein synthesis, mock reactions were performed for 7 h at 30°C, using the same conditions as above except for the omission of DNA and the use of the $^{2}H/^{15}N/^{13}C$-labelled amino acid mixture. The outside buffer was analysed by NMR without any modification except for the addition of 10% D_2O.

NMR analysis

^{15}N-HSQC spectra were recorded at 25°C on Bruker AV600 and AV800 NMR spectrometers. 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). $^{2}H–^{1}H$ exchange was assessed by ^{13}C-HSQC spectra of ubiquitin samples prepared with conventional and reduced cell-extract. Quantitative assessment of the $^{2}H–^{1}H$ exchange of $^{2}H/^{15}N/^{13}C$-labelled amino acids was achieved by integrating the ^{13}C-HSQC peak intensities measured of the outside buffer of the mock cell-free reaction and comparing the peak volumes of the amino acids to the peaks of 50 mM HEPES buffer present in the solution.

Results and discussion

Making selectively ^{15}N-labelled proteins from ^{15}N-labelled amino acids

To assess the effect of reducing the S30 extract with $NaBH_4$, we first prepared *E. coli* S30 extract and produced the *E. coli* prolyl-*cis–trans* isomerase PpiB in the conventional way (Jia et al. 2009). Figure 2 shows the

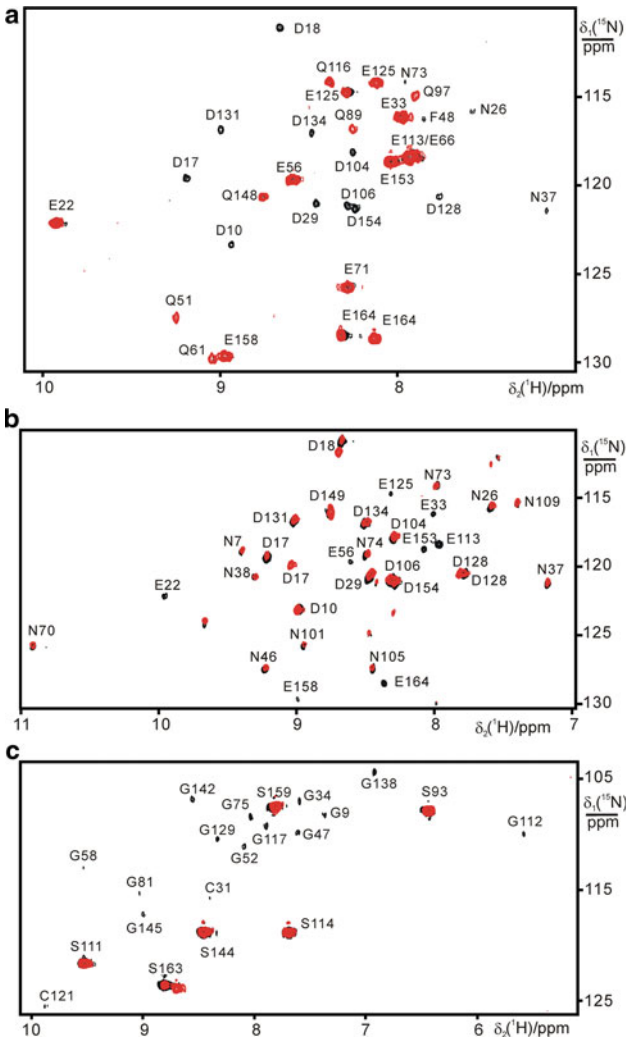

Fig. 2 Superimpositions of ^{15}N-HSQC spectra of selectively labelled *E. coli* PpiB. Spectra were recorded in 20 mM MES buffer, pH 6.5 with 2 mM DTT at 25°C on 600 (**a**, **c**) and 800 (**b**) MHz NMR spectrometers. The protein was prepared using conventional (*black peaks*) and reduced (*red peaks*) S30 extract. The samples were produced using 100 mM N-acetyl-L-glutamate (Jia et al. 2009) in the cell-free reaction. The ^{15}N-labelled amino acids added to the reaction were **a** ^{15}N-Glu, **b** ^{15}N-Asp, and **c** ^{15}N-Ser. For illustration, using conventional S30 extract led to relative cross-peak heights of **a** D17 versus E153: 45%, **b** E153 versus D17: 48%, **c** G142 versus S159: 6%. Using reduced S30 extract, the cross-peaks of D17, E153 and G142 were below the noise level of the spectra **a**–**c**, respectively. Resonance assignments taken from Kariya et al. (2000)

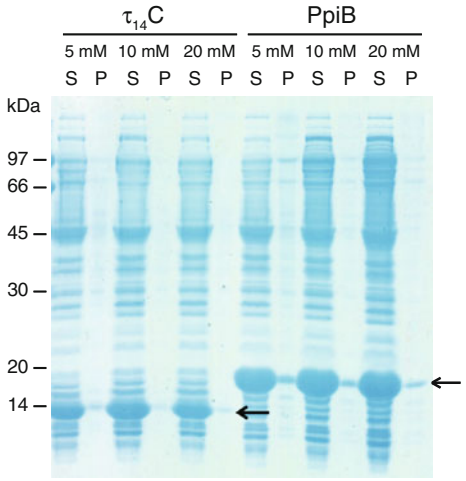

Fig. 3 Impact of different amounts of $NaBH_4$ on protein yield. Cell-free synthesis of the proteins PpiB and τ_C14 was performed using S30 extracts reduced by the final nominal concentrations of $NaBH_4$ indicated at the top. Identical volumes of reaction products were loaded onto SDS/PAGE (15%, stained with Coomassie Blue). S and P identify the fractions of supernatant and pellets, respectively. *Arrows* identify the protein bands. The positions of molecular weight markers are indicated on the left

^{15}N-HSQC spectra of selectively ^{15}N-Glu, ^{15}N-Asp, and ^{15}N-Ser labelled samples. Besides the expected cross-peaks of ^{15}N-Glu, cross-peaks of Gln, Asp, Asn, and even Phe are evident in the spectrum of Fig. 2a. In the case of ^{15}N-Asp labelling, conversion to ^{15}N-Asn and ^{15}N-Glu is apparent, and in the case of ^{15}N-Ser labelling, undesired cross-peaks of Gly and Cys are observed.

In a second experiment, we first reduced the S30 extract with $NaBH_4$. We confirmed for two proteins, PpiB and τ_C14 (Su et al. 2007), that treatment of the S30 extract with $NaBH_4$ maintained the protein expression levels (Fig. 3). Figure 2 shows that the ^{15}N-HSQC spectra of selectively ^{15}N-Glu, ^{15}N-Asp, and ^{15}N-Ser labelled samples of PpiB are greatly simplified by the use of the reduced S30 extract. In particular, the cross-peaks of Asp (in the case of ^{15}N-Glu labelling), Glu (in the case of ^{15}N-Asp labelling), and Cys and Gly (in the case of ^{15}N-Ser labelling) are strongly suppressed. To the best of our knowledge, no enzyme-specific inhibitors are known that would achieve similarly clean ^{15}N-Ser labelling.

The suppression of Gly cross-peaks in the ^{15}N-Ser labelled sample can be attributed to the inactivation of serine hydroxymethyltransferase which is a PLP enzyme. The only serious problems remaining after reduction of the S30 extract are undesired conversions from Glu to Gln, and between Asp and Asn, which can be attributed to the presence of amidotransferase, glutamine synthetase, and asparagine synthetase in the S30 extract (Yokoyama et al. 2010). These enzymes are not PLP enzymes but they can be inhibited. For example, amidotransferase is inhibited by 6-diazo-5-oxo-L-norleucine (DON; Prusiner and Stadtman 1976) and glutamine synthetase by methionine sulfoximine (Weisbrod and Meister 1973). These inhibitors markedly suppress the conversion from Glu to Gln and Gln to Glu, respectively (Figs. S1 and S2; Morita et al. 2004; Tong et al. 2008). In the case of ^{15}N-Gln labelling, use of the conventional cell-free buffer containing high concentrations of unlabelled Glu (Kigawa et al. 1995) presents an alternative, inexpensive way to suppress the appearance of cross-peaks from ^{15}N-Glu (Jia et al. 2009). Transfer of the ^{15}N-amino group from Glu to Asp and Asn can also be inhibited to some extent by the addition of 20 mM maleate (Fig. S3; Michuda and Martinez-Carrion 1970), but higher concentrations of maleate proved to reduce protein yields (data not shown).

To test the selectivity of labelling that can be achieved for all non-proline amino acids, we produced five samples of PpiB with combinatorial labelling (Wu et al. 2006), using the reduced S30 extract. With the exception of conversions between Asp and Asn, and Glu and Gln as discussed above, ^{15}N-HSQC cross-peaks of the different amino acids were observed only in those samples, where they had been added in ^{15}N-labelled form for protein production. Figure 4a displays the result for some of the amino acids most prone to isotope scrambling (Asp, Tyr, Gln, Asn, Gly, Trp) and cross-peaks of the other thirteen non-proline amino acids are shown in Fig. 4b. The cross-sections demonstrate that undesired cross-peaks were better suppressed than in corresponding experiments using conventional S30 extract (Jia et al. 2009).

Making perdeuterated proteins from perdeuterated amino acids in H_2O

Inhibiting PLP-dependent enzymes has the added benefit of suppressing the exchange of α-protons with H_2O which occurs when the amino acid is engaged in a Schiff-base with PLP (McIntosh and Dahlquist 1990; LeMaster 1990; Eliot and Kirsch 2004; Toney 2005; Berg et al. 2006). Suppression of this exchange would allow the production of perdeuterated proteins by cell-free protein synthesis in H_2O solution from perdeuterated amino acids, leaving all amides fully protonated. In contrast to proteins produced in D_2O, such samples need no subsequent exchange of labile deuterons for protons which can be impossible for proteins prone to irreversible denaturation or degradation.

^{13}C-HSQC spectra recorded of ^{2}H/^{15}N/^{13}C-labelled ubiquitin confirmed indeed that use of the reduced S30 extract greatly suppresses ^{2}H–^{1}H back-exchange. Using the signal intensities of the HEPES buffer as a concentration

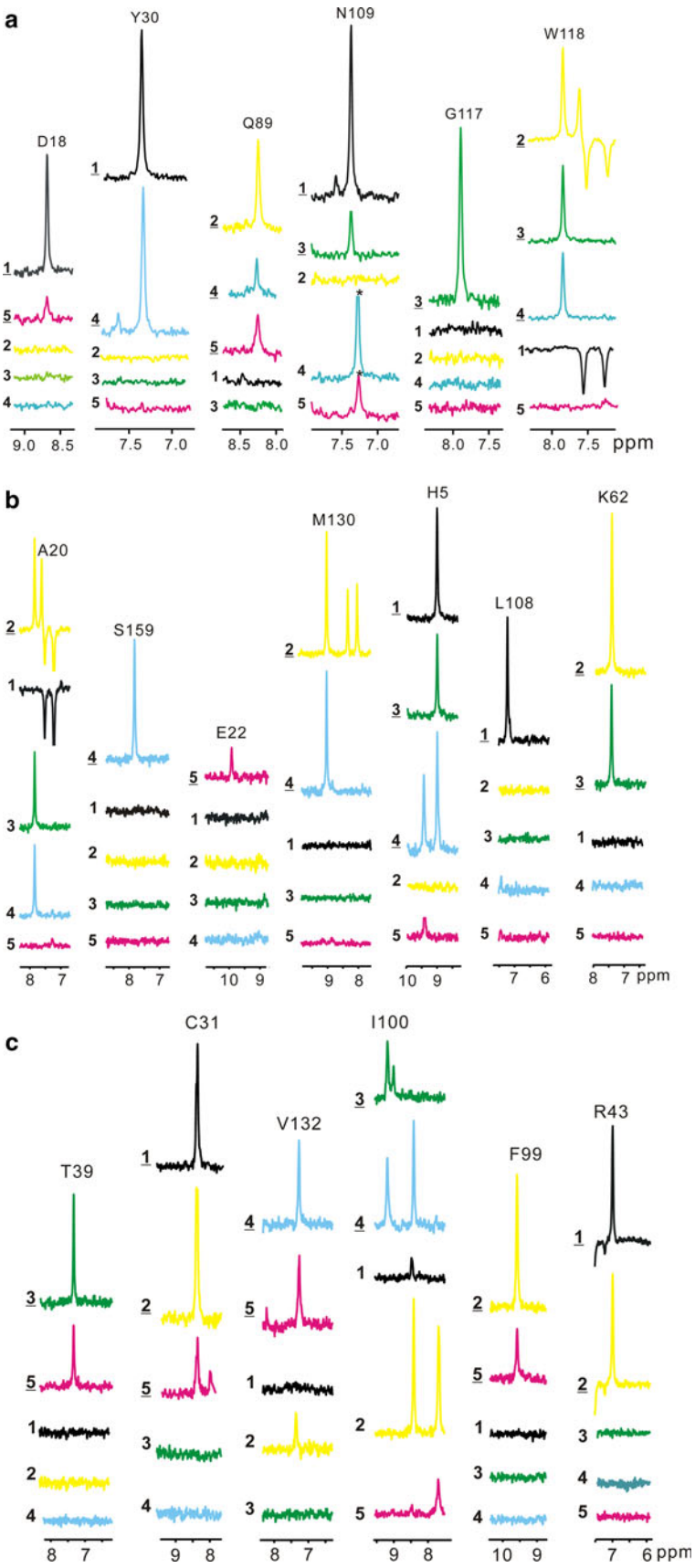

Fig. 4 Cross-sections through ^{15}N-HSQC cross-peaks of PpiB produced with combinatorial isotope labelling, illustrating the suppression of isotope scrambling by reduction of the S30 cell extract with $NaBH_4$. Samples 1–4 (*black*, *yellow*, *green*, and *blue* cross-sections) were prepared in 208 mM L-Glu buffer to suppress conversions to glutamate (Jia et al. 2009) and sample 5 (*red*) in 100 mM N-acetyl-L-Glu buffer to avoid dilution of ^{15}N-Glu. Within each panel, the cross-sections were plotted with the same scaling factor. Cross-peaks in sample 5 are relatively weak throughout in part because of lesser protein yield. Sample numbers are underlined when cross-peaks are expected because the respective amino acid was added to the cell-free reaction in ^{15}N-labelled form. ^{15}N-labelled amino acids were used as follows: 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, Met, Tyr, His, Gln, Trp (sample 4); Glu, Val, Thr, Asp, Phe, Cys, Gln (sample 5). Spectra were recorded in 20 mM MES, pH 6.5 with 2 mM DTT at 25°C. **a** Cross-peaks of Asp, Tyr, Gln, Asn, Gly, Trp. Together with Glu, these amino acids are most prone to isotope scrambling. The peaks marked with a star in samples 4 and 5 of the cross-sections of N109 belong to V139 which has a slightly different chemical shift. **b** Cross-peaks of Ala, Ser, Glu, Met, His, Leu, Lys, Thr, Cys, Val, Ile, Phe, Arg which are less prone to isotope scrambling

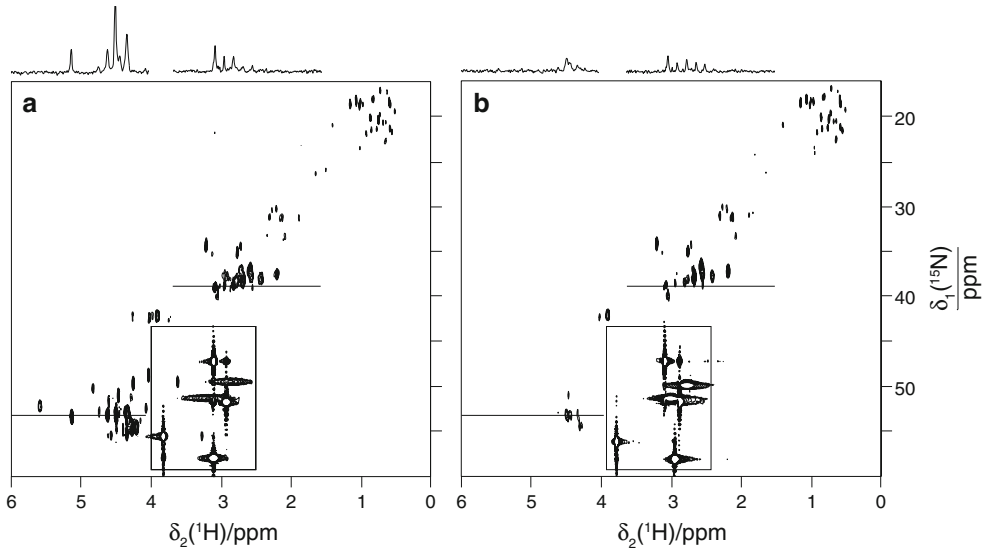

Fig. 5 ^{13}C-HSQC spectra of 0.3 mM solutions of uniformly $^{2}H/^{15}N/^{13}C$-labelled ubiquitin produced using conventional S30 extract (**a**) and reduced S30 extract (**b**) in 20 mM HEPES buffer in D_2O. The spectra were recorded at 25°C on a 600 MHz NMR spectrometer. The spectra display the same number of contour levels for the methyl groups (which are unlikely to be affected by $^{2}H–^{1}H$ back-exchange; the residual ^{1}H content of the amino acid mixture is specified as <3% by the manufacturer). Intense signals in the boxes are from the HEPES buffer at natural isotopic abundance. Cross-sections taken at the positions indicated by *lines* are shown at the *top*

standard, the conventional S30 extract produced up to 30 and 15% back-exchange at the C^{α}- and C^{β}-positions, respectively. In contrast, the use of reduced S30 extract led to much weaker (at least sixfold) cross-peaks in the $C^{\alpha}–H^{\alpha}$ region of the spectrum and an at least twofold reduction in the cross-peak intensities in the $C^{\beta}–H^{\beta}$ region (Fig. 5). Most of the intense $C^{\alpha}–H^{\alpha}$ cross-peaks originated from Asp and Glu, whereas all intense $C^{\beta}–H^{\beta}$ cross-peaks could be assigned to Asp and Asn. In addition, some of the cross-peaks in the $C^{\beta}–H^{\beta}$ region of the spectrum were associated with Lys C^{ε}. The residual back-protonation at the Asp and Asn C^{β}–positions in the experiment using reduced S30 extract indicates exchange catalysis by factors other than PLP-dependent enzymes.

To explore the impact of reduced S30 extract in a more quantitative manner, we set up cell-free expression reactions using a mixture of the 20 natural $^{2}H/^{15}N/^{13}C$ labelled amino acids. The only modification relative to the standard protocol was the omission of DNA. Following 7 h of reaction, we assessed the $^{2}H–^{1}H$ back-exchange by the appearance of ^{13}C-HSQC cross-peaks of the amino acids in the outside buffer. $^{2}H–^{1}H$ exchange detected in this experiment was pronounced only for C^{α} and C^{β} positions. As expected for $^{2}H–^{1}H$ exchange catalysed by PLP enzymes, S30 extract reduced with $NaBH_4$ produced much weaker $C^{\alpha}–H^{\alpha}$ crosspeaks than conventional S30 extract under otherwise identical conditions, leading to no more than 5% of $^{2}H–^{1}H$ exchange at any position (Table 1).

Conclusion

Reduction of cell-free extracts with $NaBH_4$ presents a widely applicable strategy for making selectively isotope labelled protein samples with minimal isotope scrambling. This opens the door to samples with exceptionally clean selective ^{15}N-labelling as well as to the production of uniformly $^{2}H/^{15}N/^{13}C$-labelled proteins in H_2O, where a very high level of perdeuteration is maintained while all amides are fully protonated. By inactivating a broad spectrum of enzymatic activities, this strategy is much simpler and cheaper than the use of S30 extracts from bacterial strains that are deficient in specific transaminases or the addition of various transaminase inhibitors to the cell-free reaction. It is also much more economical than expression systems that have been reconstituted from purified components (Shimizu et al. 2001), especially as reconstituted systems tend to deliver much lower protein yields.

Since PLP-dependent enzymes occur in all organisms, $NaBH_4$ treatment of S30 extracts can be expected to improve also the selectivity of eukaryotic cell-free systems like wheat germ extract and rabbit reticulocyte lysate. In

Table 1 ^{2}H–^{1}H exchange of the 20 natural $^{2}H/^{15}N/^{13}C$-labeled amino acids after 7 h of cell-free reaction using reduced and conventional S30 extract

Amino acid	Total concentration (mM)[a]	Amino acid with ^{2}H–^{1}H exchange (mM)[b]	^{2}H–^{1}H exchange (%)[c]	Ratio of ^{2}H–^{1}H exchange using conventional versus reduced S30 extract[d]
α-protons				
Ala	2.4	0.28	12	34
Asn	1.3	0.05	4	8
Asp	2.0	0.30	15	7
Gly	1.6	0.02	1	1
Gln	1.2	0.10	9	3
Glu	1.6	0.70[e]	45	15
Lys	1.6	[e]		
Arg	0.8	[e]		
Met	0.3	[f]		
Phe	0.3	0.08	25	6
Ser	1.0	0.05[g]	5	5
Tyr	0.5	[h]		
Trp	0.5	0.06	12	3
β-protons				
Asp	2.0	0.27	14	4
Asn	1.3	0.02	2	12

Cell-free reactions were performed at 30°C, using the same conditions as for the synthesis of PpiB except for the omission of DNA and the use of a $^{2}H/^{15}N/^{13}C$-labelled amino acid mixture. Results are shown only for those atom positions for which ^{2}H–^{1}H exchange could be detected. Specifically, no $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ cross-peaks were observed for Cys, His, Ile, Leu, Pro, Thr and Val, indicating <1% of ^{2}H–^{1}H exchange for these amino acids (<4% for His because of its relatively low concentration), irrespective of the use of conventional or reduced S30 extract

[a] Concentrations according to the manufacturer (Cambridge Isotope Laboratories)

[b] Using conventional S30 extract. Concentrations derived from cross-peak integrations of the ^{13}C-HSQC spectrum of the outside buffer of a cell-free mock reaction

[c] Third column divided by second column, expressed in percent

[d] Relative cross-peak intensities in ^{13}C-HSQC spectra of amino acids incubated with conventional versus reduced S30 extract. Dividing the values of the fourth column by those of the fifth column would yield the percentage of ^{2}H–^{1}H exchange when using reduced rather than conventional S30 extract

[e] The $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ cross-peaks of glutamate, lysine, and arginine overlapped in the ^{13}C-HSQC spectrum; the ^{2}H–^{1}H exchange of glutamate was calculated on the assumption that only a negligible fraction of the cross-peak intensity was contributed by lysine and arginine, as indicated by the results by Etezady-Esfarjani et al. (2007)

[f] The cross-peak of methionine overlapped with t_1-noise from a HEPES cross-peak. ^{2}H–^{1}H exchange of greater than 10% would have been detectable

[g] The $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ cross-peaks of serine and tyrosine overlapped in the ^{13}C-HSQC spectrum. The ^{2}H–^{1}H exchange of serine was calculated on the assumption that only a negligible fraction of the cross-peak intensity was contributed by tyrosine as reported by Etezady-Esfarjani et al. (2007)

our hands, reduced and frozen *E. coli* S30 extracts store as well as conventional extracts.

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