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ARTICLE

Cell-free protein synthesis of perdeuterated proteins for NMR studies

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Abstract Cell-free protein synthesis protocols for uniformly deuterated proteins typically yield low, non-uniform deuteration levels. This paper introduces an *E. coli* cell-extract, D-S30, which enables efficient production of proteins with high deuteration levels for all non-labile hydrogen atom positions. Potential applications of the new protocol may include production of proteins with selective isotope-labeling of selected amino acid residues on a per-deuterated background for studies of enzyme active sites or for ligand screening in drug discovery projects, as well as the synthesis of perdeuterated polypeptides for NMR spectroscopy with large supra-molecular structures. As an illustration, it is demonstrated that the 800-kDa chaperonine GroEL synthesized with the D-S30 cell-free system had a uniform deuteration level of about 95% and assembled into its biologically active oligomeric form.

Keywords Back protonation of deuterated amino acids · Cell-free protein synthesis · Deuterated proteins · GroEL · Isotope labeling

Abbreviations

CECF	continuous-exchange cell-free
FKBP	FK506-binding protein
MS	mass spectrometry
MWCO	molecular weight cut-off
RNAP	RNA polymerase.

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Introduction

Cell-free protein synthesis is of special interest for the preparation of protein samples with non-uniform isotope labeling for NMR spectroscopy. In particular, it provides for efficient incorporation of selectively labeled amino acids into polypeptide chains in situations where in vivo protein expression typically results in isotope scrambling or isotope dilution (Kigawa et al. 1995; Ozawa et al. 2004; Waugh 1996), and its use can extend to cytotoxic proteins, such as proteases or apoptosis-related proteins (Adrain et al. 2006). Systems based on *E. coli* or wheat germ cell extracts in continuous exchange reaction chambers are widely used, with yields of several milligrams of protein per milliliter of reaction mixture (Klammt et al. 2004; Spirin et al. 1988; Vinarov et al. 2004). These fundamental procedures are increasingly being supplemented by eukaryotic cell-free systems generated from insect cells (Ezure et al. 2006; Katzen and Kudlicki 2006; Wakiyama et al. 2006) or hybridoma cell-lines (Mikami et al. 2006), which allow further studies of post- and co-translational events such as *N*-myristoylation (Sakurai et al. 2006).

This paper addresses specific applications of in vitro synthesis for the production of perdeuterated proteins. Work on a perdeuterated background is of potential interest for studies of protein functions using residue-selective stable isotope labeling. Furthermore, although most of the proteins produced so far with the aforementioned cell-free expression protocols are small monomeric structures,

in vitro expression with stereo-array isotope labeling (SAIL; Kainosho et al. 2006; Sugimori et al. 2004) has generated interest in cell-free production of larger proteins, and it has been well documented that high levels of deuteration are indispensable for solution NMR studies of polypeptides in structures of sizes above 40 kDa (LeMaster 1989; Pachter et al. 1992). With in vivo expression in ^2H_2O-based medium, perdeuterated proteins are, however, usually obtained only after lengthy "training" of the cells, *albeit* typically with significantly lower expression yields than in H_2O-based growth media. Therefore, we felt that it is of interest to explore the use of cell-free synthesis also for the production of perdeuterated proteins. In this study, we describe a protocol that achieves this goal and demonstrate its use with the synthesis of the 800-kDa *E. coli* chaperonine GroEL, which is an oligomeric protein with 14 identical subunits (Xu et al. 1997).

Materials and methods

Preparation of S30 and D-S30 extract

For the preparation of S30 extract, the *E. coli* strain BL21 Star (DE3; Invitrogen) was used, since the cell extract from this strain showed a better performance than the S30 extract obtained from regular BL21 (DE3) or BL21 (DE3) RIL strains (Kigawa et al. 2004). The production of S30 extract was based on previously reported protocols (Kigawa et al. 2004; Torizawa et al. 2004), with modifications that increased the efficiency of protein synthesis. Thereby, 9 l of incomplete rich medium containing 10 g/l of yeast extract, 1% w/v glucose, 40 mM KH_2PO_4 and 165 mM K_2HPO_4 were inoculated with 40 ml of overnight culture. The cells were harvested at an early phase of exponential growth (OD_{600} of 0.7), and were then washed three times with 400 ml of ice-cold S30 buffer (7 mM β-mercaptoethanol, 1 mM dithiothreitol, 14 mM magnesium acetate, 60 mM potassium acetate, 10 mM Tris-acetate at pH 8.2). Cell-breakage was carried out with a single passage of the cell suspension (0.8 g/ml) in S30 buffer through a French-Press at 15,000 psi, followed by two rounds of centrifugation of the cell-lysate at 30,000 × *g* at 4°C.

In order to increase the translation efficiency, the cell extract thus obtained was supplemented with purified ribosomes that had been isolated from 9 l of *E. coli* BL21 Star cell culture (DE3; Invitrogen) grown in incomplete rich medium. Ribosome purification was based on ultracentrifugation (115,000 × *g*, 17 h at 4°C) and a sucrose cushion approach (Schaffitzel and Ban 2007). 50 mg of purified ribosomes were thus obtained per liter of *E. coli* cell culture.

For removal of the endogenous mRNAs and their translation products, 1 ml of freshly obtained cell-lysate was incubated for 60 min at 25°C with 155 µl of a solution containing 13 mM ATP, 40 µM of each of the 20 proteinogenic amino acids, 4 mM DTT, 9 mM magnesium acetate, 85 mM phosphoenolpyruvate, 20 units of pyruvate kinase and 290 mM Tris-acetate at pH 8.2. After incubation, the extract was dialyzed three times against 1 l of S30 buffer at 4°C, followed by an additional overnight dialysis against 2 l of S30 buffer at 4°C using a Spectra/pore membrane with a molecular weight cut off (MWCO) of 3,000 Da. These extensive dialysis steps ensured complete removal of endogenous amino acids from the cell extract. Using a dialysis step against polyethylene glycol 8000 (PEG-8000) in /S30 buffer (50% w/w), the final volume of the extract obtained from 9 l of cell culture was reduced to 15 ml, and after additional centrifugation for 10 min at 4,000 × *g* and 4°C, the S30 extract was stored at –80°C until further use.

For the preparation of D-S30 extract, 5 ml aliquots of S30 extract were dialyzed against S30 buffer in ^2H_2O, using a centrifugal filter device (MWCO 10,000 Da), and the buffer exchange against an equal volume of deuterated S30 buffer was repeated six times.

Expression and purification of T7 RNA polymerase

Expression and purification of T7 RNA polymerase was based on using the pT7-911 vector, which is derived from pQE-8 (Qiagen) encoding the T7 RNA polymerase (RNAP) gene with a poly-His_6 purification tag at the N-terminus. RNAP was expressed in *E. coli* cells BL21 (DE3) (Stratagene), using a standard procedure with induction of protein expression by the addition of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cell breakage was carried out by several passages through a French-Press at 20,000 psi, and protein purification was carried out by affinity chromatography using a Ni–NTA–agarose resin column followed by size exclusion chromatography.

Cell-free protein synthesis

Analytical-scale cell-free synthesis of the FK 506-binding protein (FKBP) and of GroEL was carried out in batch mode. In order to optimize the reaction conditions of temperature, salts and amino acid concentrations, the cell-free reaction setup was first carried out using unlabeled amino acids (Cambridge Isotope Laboratories, Inc., CIL). The optimized conditions were then used for preparative-scale synthesis of U-^2H, U-^{15}N-labeled samples of FKBP and GroEL, using a [U-^2H 98%, U-^{15}N 98%]- amino acid mixture (CIL).

The genes of FKBP and GroEL were cloned into the cell-free vectors pIVEX 2.3d and pIVEX 2.4d (Roche). Depending on the overall reaction yield and the isotope consumption, the large-scale reaction was carried out either in the batch mode or by continuous-exchange cell-free (CECF) protein synthesis. The nutrient compositions used for the batch and CECF modes are given in Table 1. The ratio of reaction volume and feeding volume in the CECF setup was 10:1. For the production of deuterated proteins, all the substrates for the cell-free protein synthesis reaction were prepared in ^2H_2O. In order to remove residual water prior to the preparation of the substrates in ^2H_2O, the stable salts (Table 1) were heated at 200°C for 2 h before they were dissolved in ^2H_2O. The reaction mixture was incubated at 30°C and 450 rpm either for 5 h (batch mode), or for 17 h (CECF mode), using a tabletop thermomixer (Eppendorff).

Protein purification

Purification of FKBP was based on affinity chromatography using a Ni–NTA–agarose resin column and a linear gradient of imidazole. After the cell-free reaction, the reaction and feeding volumes were collected and centrifuged for 10 min at 15,000 g. The supernatant was loaded on the Ni–NTA–agarose resin column equilibrated with buffer A (30 mM imidazole, 500 mM NaCl, 20 mM sodium phosphate, pH 7.2) and the column was washed with three volumes of this buffer. FKBP was eluted from the column with a linear gradient of imidazole (0-500 mM, 5 column volumes) in buffer A, together with RNAP. An additional size exclusion chromatography was carried out to separate RNAP from FKBP, using a Superdex 75 prep grade column (GE Healthcare) equilibrated in NMR buffer (20 mM sodium phosphate, pH 6.2). The fractions containing FKBP were pooled and concentrated to a final volume of 500 µl. Typically; protein concentrations of about 500 µM (5 mg of protein) were obtained from a 4 ml CECF protein synthesis reaction.

Table 1 Composition of the reaction mixture (1 ml) and the feeding mixture (10 ml) used for CECF protein synthesis[a]

Components	Reaction mixture	Feeding mixture
S30-extract (OD_{280}, 200)	330 µl	–
Plasmid DNA	10 µg/ml	–
T7-RNA polymerase	170 µg/ml	–
E. coli tRNA	240 µg/ml	66 µg/ml
Mixture of 20 amino acids	1.5 mM (each)	1.5 mM (each)
Creatine kinase	83 µg/ml	–
Phosphocreatine	84 mM	84 mM
ATP	1.27 mM	1.27 mM
CTP, GTP, UTP	0.9 mM (each)	0.9 mM (each)
cAMP	0.67 mM	0.67 mM
Folinic acid	36 µg/ml	36 µg/ml
DTT	2.1 mM	2.1 mM
Tris-acetate, pH 8.2	3 mM	3 mM
Hepes-KOH, pH 7.5	60 mM	60 mM
Ammonium acetate	29 mM	29 mM
Magnesium acetate	11.2 mM	11.2 mM
Potassium acetate	20 mM	20 mM
Potassium glutamate	210 mM	210 mM
Polyethyleneglycol 8000	4% (w/v)	4% (w/v)
Sodium azide	0.025% (w/v)	0.025% (w/v)

[a] In the batch setup, the reaction was carried out in the reaction mixture for 4–5 h at 30°C and 450 rpm

For the purpose of comparative studies, we also prepared in vivo deuterated GroEL, based on previously reported isolation methods (Fiaux et al. 2002; Xu et al. 1997). Cells carrying a plasmid for GroEL overexpression were grown under ampicillin control in ^2H_2O -based minimal medium, and overexpression was induced with IPTG. 2–3 g of harvested cells were resuspended in 30 ml of extraction buffer (100 mM TrisCl, 1mM DTT, 0.1 mg/ml DNase I, pH 8.0) and lysed by sonication followed by centrifugation for 30 min at 30,000 × *g* and 4°C. At this point, only a single step of ammonium sulphate precipitation (40% w/v) was carried out, and after another centrifugation step the supernatant was applied to a HiPrep 26/10 desalting column (GE Healthcare). This was followed by anion exchange chromatography using a DEAE-Sepharose fast flow column equilibrated in buffer B (50 mM TrisCl, 2 mM DTT, pH 7.2). Prior to elution, the column was washed with three column volumes of buffer B, and GroEL was eluted using a linear gradient of NaCl (0–500 mM, 5 column volumes) in buffer B. The fractions containing GroEL were concentrated and loaded on a Superdex 200 prep grade column equilibrated with NMR buffer (25 mM potassium phosphate, 20 mM KCl, pH 6.2).

For in vitro synthesized GroEL, the ammonium sulphate precipitation step was omitted. Although similar elution profiles from the gel filtration column were observed for in vivo and in vitro produced GroEL, insoluble aggregates were immediately formed upon addition of 40% w/v ammonium sulphate to the in vitro reaction mixture. Therefore, an additional anion exchange chromatography step was carried out after the initial DEAE-Sepharose fast flow column and the following desalting step, using a high resolution Resource Q column (GE Healthcare) equilibrated in buffer B. GroEL was eluted from the column using a linear gradient of NaCl (0–500 mM, 10 column volumes). Finally, fractions containing GroEL were concentrated and subjected to size exclusion chromatography, as described for the in vivo expressed GroEL.

Collection of NMR data

All NMR experiments with FKBP were recorded at 25°C on a Bruker DRX600 spectrometer equipped with a standard triple resonance probehead. The protein concentration was adjusted to 0.8 mM. For the 3D ^{15}N-resolved [^{1}H,^{1}H]-TOCSY experiment with a mixing time of τ_m = 60 ms, eight transients were added with an interscan delay of 1 s, resulting in a total measurement time of 39 h. 1024 complex points were recorded with an acquisition time of 131 ms, and prior to Fourier transformation the FID was multiplied with a 75°-shifted sine bell and zero-filled to 2048 complex points. In the ω_1(^{1}H)-dimension, 95 complex points were measured, with a maximal evolution time of 12 ms, and the data was multiplied with a 75°-shifted sine bell (De Marco and Wüthrich 1976) and zero-filled to 256 complex points before Fourier transformation. In the ω_2(^{15}N)-dimension, 42 complex points were measured, with a maximal evolution time of 21 ms, and the data was multiplied with a 75°-shifted sine bell and zero-filled to 128 complex points before Fourier transformation. For the 2D [^{1}H,^{1}H]-NOESY experiments with a mixing time of τ_m = 100 ms, 128 transients were added with an interscan delay of 1 s, resulting in a total measurement time of 20 h. About Thousand and twenty four complex points were recorded with an acquisition time of 128 ms, and prior to Fourier transformation the FID was multiplied with a 75°-shifted sine bell and zero-filled to 2048 complex points. In the ω_1(^{1}H)-dimension, 256 complex points were measured, with a maximal evolution time of 32 ms, and the data was multiplied with a cosine function and zero-filled to 512 complex points before Fourier transformation.

For GroEL produced by cell-free expression, a 2D [^{15}N,^{1}H]-CRIPT-TROSY spectrum was recorded at 35°C on a Bruker Avance900 spectrometer equipped with a standard triple resonance probehead. The protein concentration was 0.7 mM in monomers, and the transfer time was T = 1.4 ms (Fiaux et al. 2002). 4048 transients were added with an interscan delay of 300 ms, resulting in a total measurement time of 4 days. In the ω_2 (^{1}H)-dimension, 1024 complex points were recorded with an acquisition time of 81 ms. Prior to Fourier transformation the FID was multiplied with a cosine function and zero-filled to 2048 complex points. In the ω_1 (^{15}N)-dimension, 100 complex points were measured, with a maximal evolution time of 20 ms, and prior to Fourier transformation the data was multiplied with a 20°-shifted sine bell and zero-filled to 256 complex points.

For GroEL prepared in vivo, a 2D [^{15}N,^{1}H]-CRIPT-TROSY experiment was recorded with the same conditions as for the cell-free preparation, except for the following: the experiment was recorded on a DRX750 spectrometer, the protein concentration was 1.9 mM in monomers, 512 transients were added, the acquisition time was 97 ms, in the ω_1 (^{15}N)-dimension the maximal evolution time was 22 ms, and the total measurement time was 12 h.

In all experiments, the baseline was corrected using the IFLAT method (Bartels et al. 1995) in the direct dimension, and with polynomials of 2nd order in the indirect dimension.

Results and discussion

Cell-free expression of GroEL in H_2O-based growth medium

Cell-free synthesis of GroEL was carried out using the batch mode approach, which is cost-effective for the expression of large amounts of protein due to the low amount of amino acids needed (Table 1). This is an important aspect, since about 30 mg of protein are required for an NMR sample with a GroEL concentration of 1 mM in monomers. About 400 μg of GroEL were produced from 1 ml of reaction mixture (Fig. 1a and b), of which about 55% were accessible in soluble form, as shown by analytical ultra-centrifugation. The formation of the native multimeric form was confirmed by analytical size exclusion chromatography (Fig. 1c), which showed that the retention time and the elution profiles were almost identical to those of in vivo expressed GroEL.

Attempts to produce perdeuterated proteins using H_2O-based S30 extract

As a first approach to cell-free production of perdeuterated proteins, the standard cell-free protein synthesis protocol (Torizawa et al. 2004) was applied, using a mixture of the 20 proteinogenic amino acids with U-98% deuteration as the amino acid source (Table 1). The relative expression level with the deuterated amino acids was about 95% of the yield in cell-free synthesis of GroEL using unlabeled amino acids. However, mass spectroscopy of the protein showed a 0.5% smaller mass than expected for the fully perdeuterated protein, which could have been due to the incorporation of non-labeled amino acids present in the S30 extract. To eliminate this potential source of unlabeled amino acids, the protocol used for the preparation of S30 extract was supplemented with an additional size exclusion chromatography step (Torizawa et al. 2004). Absence of residual amino acids in the resulting S30 extract was confirmed by the observation that immune-blotting assays for protein synthesis in reaction mixtures without amino acid supplementation did not show any detectable bands of newly synthesized protein. Nonetheless, the deuteration

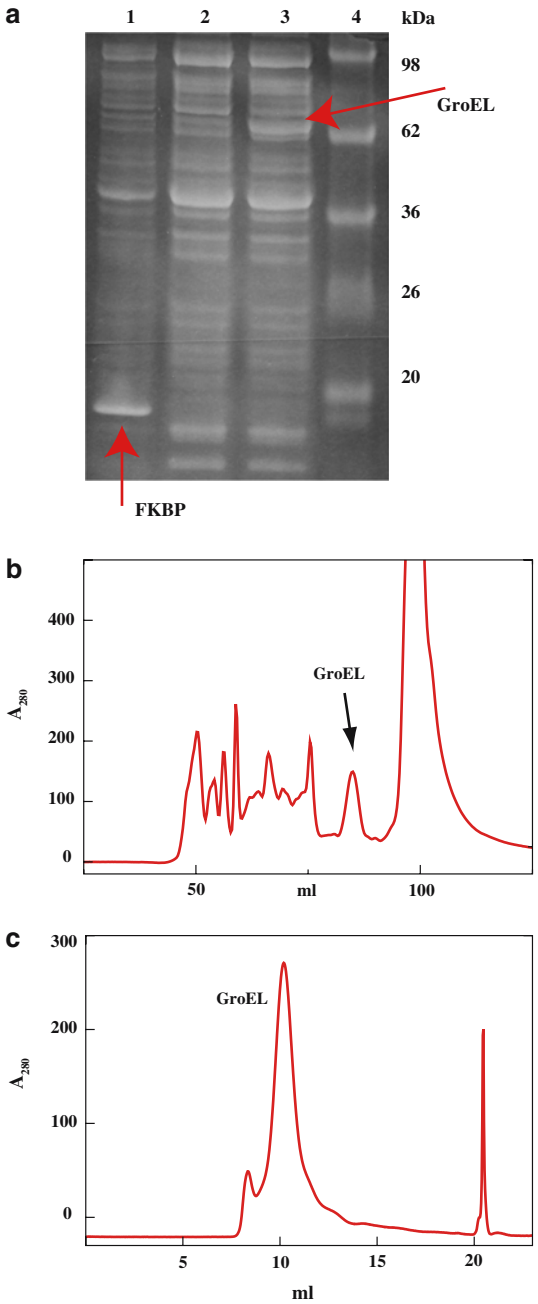

Fig. 1 Cell-free protein synthesis of GroEL and FKBP at natural isotope distribution. (**a**) Translation efficiency of cell-free protein synthesis monitored by SDS-Page. Lanes: 1, FKBP using CECF protein synthesis, the yield is 1.5 mg/ml reaction mixture; 2, negative control; 3, GroEL synthesized by batch mode cell-free proteins synthesis, the yield is 300 μg/ml reaction mixture; 4, protein ladder with indication of molecular weights in kDa. (**b**) Anion exchange chromatography for the purification of cell-free synthesized GroEL. The protein elutes as a single peak, identified by an arrow. (**c**) Analytical size exclusion chromatography of GroEL. The elution volume of 10.4 ml corresponds to a particle size larger than 400 kDa (column specification)

level of the protein expressed with perdeuterated amino acids did not increase significantly. 1D ^{1}H-NMR spectra of a sample of 31 mg of deuterated GroEL prepared in a 100 ml reaction volume using the batch mode indicated a high degree of back-protonation at H^{α}- positions, and to a lesser extent also at H^{β}- positions. In contrast, the high-field region of the ^{1}H NMR spectrum showed a deuteration level of about 95% for the methyl groups of the protein (data not shown).

At this point we turned to the 110-residue FK506 binding protein (FKBP) for more detailed investigations of the α- and β-protonation levels in proteins from cell-free production with perdeuterated amino acids, since for FKBP the chemical shift assignments are known (Rosen et al. 1991; Xu et al. 1993). A reaction volume of 4 ml in a CECF setup produced 6.3 mg of FKBP, resulting in a 1.1 mM NMR sample. NMR experiments confirmed the observation of partial back-protonation at the α- and β-positions (Fig. 2), showing that it is exchange of H^{α} and H^{β} with bulk water during the cell-free polypeptide synthesis system that prevents perdeuteration of the protein. Cell-free synthesis of several other soluble and membrane proteins in both batch and CECF setups yielded similar results to those with FKBP. Semi-quantitative analysis of 2D [^{1}H,^{1}H]-NOESY and 3D ^{15}N-resolved [^{1}H,^{1}H]-TOCSY spectra recorded with FKBP further revealed that the back-exchange is amino acid-specific, and that the 20 amino acids can be classified in four groups with different relative back-protonation levels (Table 2). At the α-position, Asp, Asn, Glu and Gln show high back-protonation, Gly, Ser, Cys, Ala, Phe, Trp and Tyr are a second group with intermediate back-protonation, Val, Ile, Leu Lys and Arg have a low degree of back-protonation, and Thr, His and Met show almost no back-protonation. At the β-position, Asp, Asn, Gln and Glu have high back-protonation, Ala, Phe and Tyr show low β back-protonation (Fig. 2), and no back-protonation was detected for the other amino acids with the presently used methods.

Preparation of deuterated S30 extract (D-S30) for cell-free synthesis

In cell-free synthesis with the batch setup, 33% of the reaction volume is comprised of S30 extract, which was thus a prime target for modifications aimed at reduced back-protonation. In principle, deuterated S30 extract could be obtained from cells grown in $^{2}H_2O$-based medium. However, since the harsh growth conditions in $^{2}H_2O$ reduce the overall fitness of bacterial cells, we would expect the activity of such cell-extract to be reduced. Further, since about 18 l of $^{2}H_2O$ would be needed for obtaining 15 ml of cell-extract, this approach would not be cost-effective. We therefore chose the alternative of exchanging $^{2}H_2O$ into S30 extract that had been prepared on a H_2O-basis, either by filtration, dialysis or

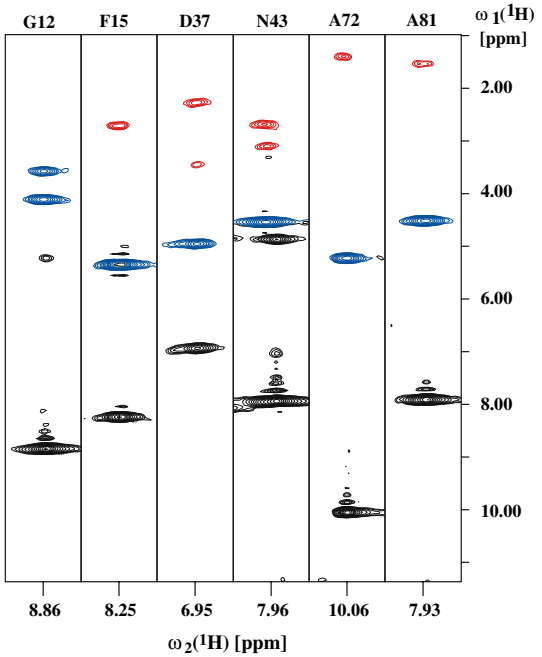

Fig. 2 Observation of back-protonation during the incorporation of perdeuterated amino acids by cell-free protein synthesis of FKBP in H_2O-based reaction mixture monitored with 3D ^{15}N resolved [^1H,^1H]-TOCSY experiments (τ_m = 60 ms). Data are shown for six residues, where the H^N–N–H^N cross peaks are black (these serve as a reference with relative intensity corresponding to 100% occupancy of the two correlated proton sites), the H^N–N–H^α peaks are blue, and the H^N–N–H^β peaks red. The intensities of the blue and red peaks manifest the extent of back-protonation at the H^α and H^β sites, respectively. The NMR data were processed with the software PROSA (Güntert et al. 1992)

Table 2 Amino acid-dependent back-protonation at the α- and β-positions during cell-free protein synthesis using perdeuterated amino acids in H_2O-based culture medium

Amino acids	Relative degree of α- and β -back-protonation[a]
Asp, Asn, Gln, Glu	Very high
Ala, Cys, Gly, Phe, Ser, Trp, Tyr	High
Arg, Ile, Leu, Lys, Val	Medium
Thr, His, Met	Low

[a] The classification is based on semi-quantitative analysis of 3D ^{15}N-resolved [^1H,^1H]-TOCSY and 2D [^1H,^1H]-NOESY spectra

lyophilization. In all assays, the activity of the resulting ^2H_2O–S30 extract was compared to the activity of standard S30 extract.

High activity of the cell extract in ^2H_2O with reasonable buffer exchange costs was achieved using an optimized filtration procedure. In a series of assays, H_2O was replaced by ^2H_2O using different filtration devices covering molecular weight cutoffs (MWCO) between 3,000 and 50,000 Da. The resulting extracts were evaluated by their protein synthesis efficiencies as monitored by Western blotting. Optimal synthesis efficiency was obtained with a MWCO of 10,000 Da. MWCO values below 10,000 resulted in the formation of larger amounts of precipitates, which could not be resuspended, and MWCO values above 10,000 resulted in reduced extract activity. The maximal translation efficiency of the D-S30 extract was about 65% of that of the conventional S30 extract. For preparative-scale production of D-S30 cell extract we used six filtration cycles with a dilution factor of 2, resulting in a ^2H_2O level above 98%. A higher number of filtration cycles resulted in reduced translation efficiency of the D-S30 extract. In spite of inevitable residual H_2O content in the other components of the cell-free reaction setup (Table 1), the final H_2O concentration in the D-S30-based reaction mixture was less than 5%.

Comparable results to those from the adopted filtration procedure were achieved with conventional dialysis, resulting in 60–70% of the activity of S30 extract, but due to the large volume of ^2H_2O needed for complete buffer exchange, this approach is economically not viable.

Lyophilization turned out to be inadequate, since only partial resuspension of the lyophilized extract was achieved, with nearly zero remaining activity of the cell-extract. This is probably due to the fact that several steps of freezing followed by lyophilization of cell-extract are required to remove residual water, and each lyophilization had to be carried out for several hours.

The D-S30 extract was supplemented with purified ribosomes prior to the final filtration step, which further increased the overall efficiency of the synthesis reaction to about 85% of the efficiency of the conventional S30 extract without ribosome supplementation (see Material and methods).

Comparison of deuteration levels obtained with S30 and D-S30 extract

The Figs. 3 and 4 show comparisons of 2D [^1H,^1H]-NOESY data of FKBP produced in vitro using either the conventional S30-extract or the new D-S30 extract. All the spectra were recorded on a 600 MHz spectrometer with a mixing time of 100 ms, and processed identically. The protein concentration was adjusted to 800 µM as determined by UV absorption spectra. The high level of back-protonation in the protein prepared with conventional S30-extract is manifested by the appearance of numerous H^N–H^α and H^N–H^β cross peaks (Figs. 3 and 4b). Most of the H^N-H^α cross-peaks in Fig. 4b originate from Asp (Fig. 3a), Asn, Glu and Gln residues, which also show a high tendency for back-protonation in the β-positions. In addition, the back-protonation of the Ala methyls is rather

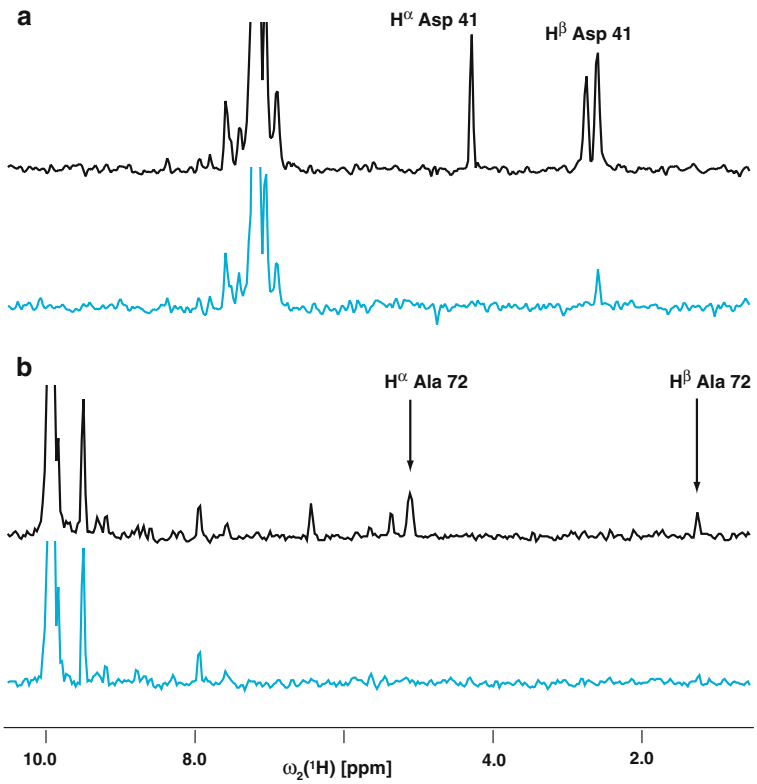

Fig. 3 Amino acid-specific back-protonation monitored by comparison of 1D cross-sections taken from 2D [^{1}H,^{1}H]-NOESY experiments recorded with FKBP at 600 MHz with a mixing time of 100 ms. (**a**) Asp 41. (**b**) Ala 72. Color code: black, sample prepared in H_2O-based S30 cell extract; cyan, synthesis in D-S30 cell extract. In both experiments, U-^{2}H 98%, U-^{15}N 98% amino acids were used in the reaction mixture

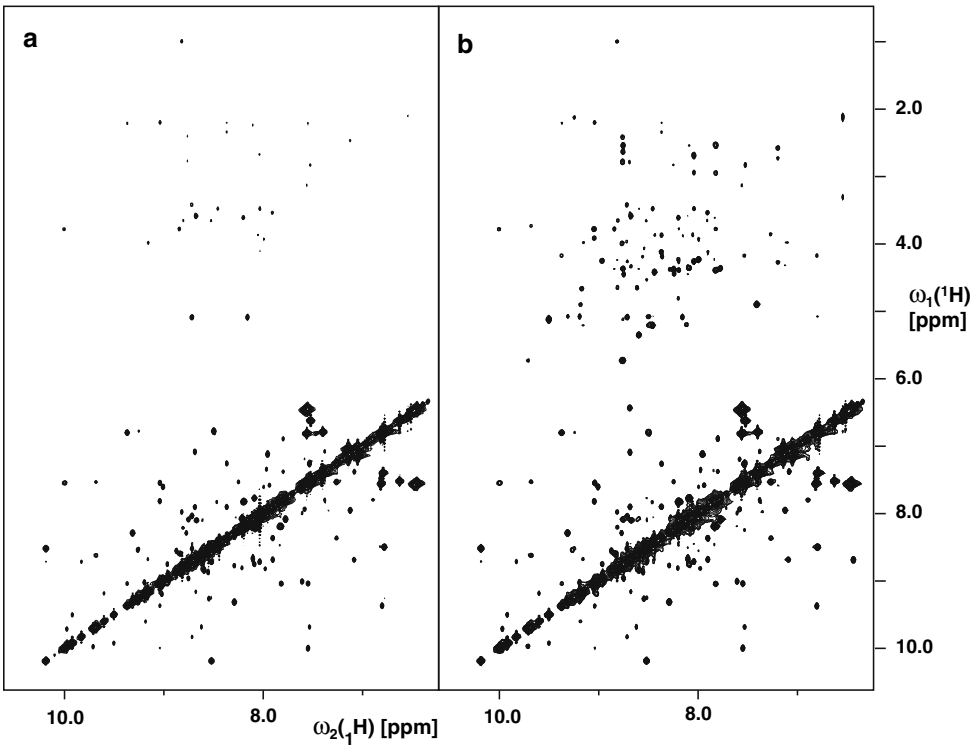

Fig. 4 Back-protonation of perdeuterated amino acids during in vitro synthesis of FKBP monitored by comparison of the 600 MHz 2D [^{1}H,^{1}H]-NOESY spectra of FKBP produced in vitro using either D-S30 cell extract (**a**) or H_2O-based S30-extract (**b**). The protein concentration was 800 µM in both samples, the measurements were carried out at 23°C with a mixing time of 100 ms, and the two spectra were recorded and processed identically

striking (Fig. 3b). The reduced back-protonation in the FKBP preparation from the D-S30 extract is reflected by the dramatically reduced peak intensities in the cross sections of Fig. 3. In Fig. 4a the spectral region [ω_1 = ω_2 = 6.0–10.3 ppm], which contains amide proton-amide proton cross peaks, is identical to Fig. 4b, whereas the

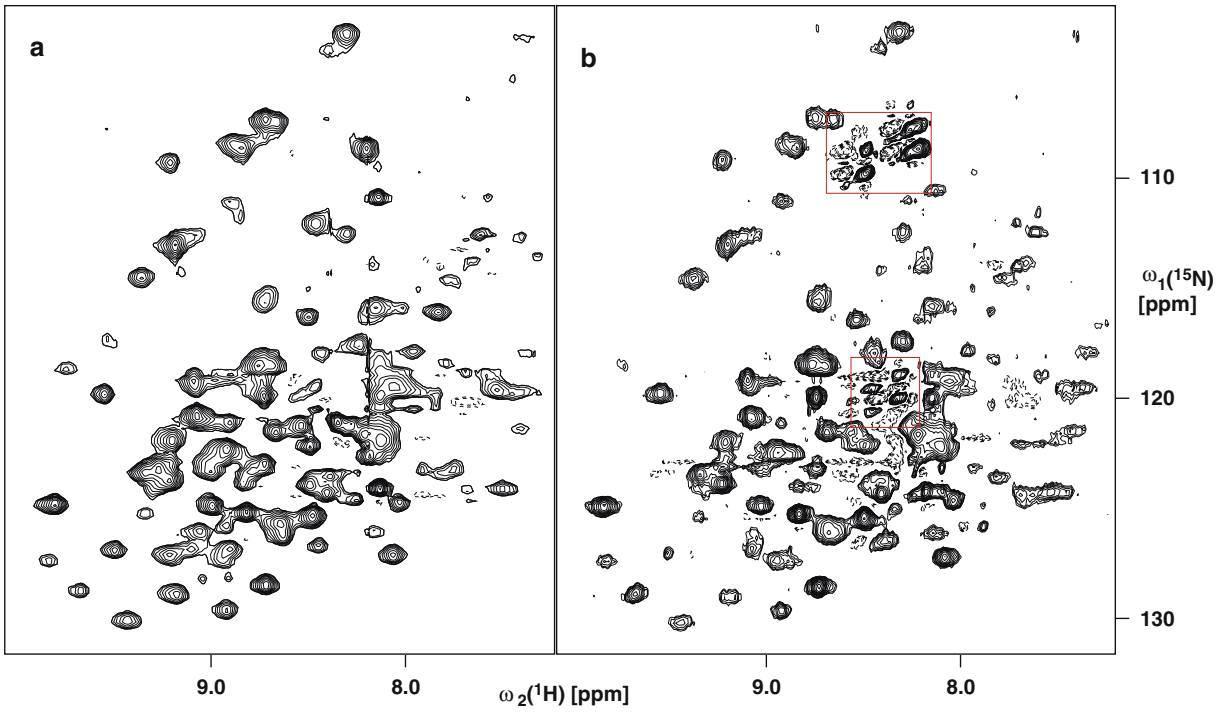

Fig. 5 2D [^{15}N,^{1}H]-CRIPT-TROSY NMR spectra (Fiaux et al. 2002; Riek et al. 2002) of two different samples of uniformly ^{2}H,^{15}N-labelled GroEL. (**a**) Protein expressed in vivo in *E. coli* cells. (**b**) Protein synthesized in vitro with D-S30 extract. Two spectral regions highlighted in spectrum (**b**) by red rectangles identify local differences between the two spectra that are discussed in the text

much lower population of H^N–H^α and H^N-H^β cross peaks in the remainder of the spectrum is readily apparent. There is some residual back-protonation at the β-positions of Asp (Fig. 3a) and Asn, but this is also much lower than with the conventional S30 extract.

GroEL synthesis with D-S30 extract

The translation efficiency of the new D-S30 extract for the synthesis of GroEL with unlabeled or with perdeuterated amino acids was similar. The elution profiles were similar to those shown in Fig. 1 for protonated GroEL. A MS analysis of GroEL synthesized from deuterated amino acids in D-S30 extract showed a deuteration level of about 95%. 2D [^{15}N,^{1}H]-CRIPT-TROSY spectra of GroEL synthesized with the D-S30 extract and of GroEL prepared by in vivo protein expression show high similarity of the two samples (Fig. 5), indicating that proper assembly of the cell-free synthesized GroEL monomers into the biologically active 14-mer was achieved.

An observation of potential biological interest resulted for the peaks contained in the red boxes of Fig. 5b, which have higher intensity than the signals at the same chemical shifts in the in vivo produced GroEL (Fig. 5a). To follow this up, we used the same gene construct of GroEL in the pIVEX 2.3d vector for both in vivo expression and cell-free synthesis of unlabeled GroEL. MALDI mass spectroscopy of the purified protein samples indicated that the mass of the in vitro synthesized GroEL is 116 Da higher than that of the in vivo expressed protein. The terminal residue in the GroEL sequence is methionine, and cleavage of this residue would give a mass reduction by 131 Da. Since the deviation of the molecular mass difference of 15 Da is within the expected error range of ± 20 Da, we speculate that the absence of the terminal Met in the in vivo produced GroEL results in reduced flexibility of the C-terminal polypeptide segment and concomitant line-broadening of the NMR signals.

Conclusions

The presently described protocol for cell-free synthesis of perdeuterated proteins based on the use of deuterated D-S30 cell extract yields 95% deuteration of the non-labile proton positions, with similar expression levels as the H_2O-based S30 cell extract. With this approach, in vitro synthesis of perdeuterated proteins should thus be economically viable. In addition to the production of perdeuterated proteins, the protocol may be adaptable for more sophisticated isotope labeling schemes, which are not readily accessible by other techniques. For example, selective incorporation of individual ^{1}H, ^{13}C ,^{15}N-labeled

amino acid types into proteins on a uniform ^{2}H, ^{12}C, ^{14}N-background could open new perspectives for active site screening of enzymes and receptor proteins, with practical applications in drug discovery and drug design projects. The approach used here with *E. coli* cell extract should in principle also be applicable with other cell-free systems, for example, with wheat germ or insect cell extract for the production of eukaryotic proteins.

As mentioned above, the ability to produce proteins with uniform high-level deuteration of all non-labile and labile hydrogen positions is of interest for many potential applications. A limitation of the D-S30-based in vitro expression system is that it cannot yield selective deuteration of only the non-labile protons. This type of deuterium labeling would be of interest in all situations where part of the labile proton positions cannot be exchanged with the solvent when dissolving the perdeuterated protein in H_2O (Wüthrich 1986). Our studies of back-protonation of deuterated amino acids during cell-free synthesis in H_2O-based cell extract (Figs. 2–4) may, however, provide a starting platform for the future design of protocols enabling in vitro synthesis of proteins with selective deuteration of only the non-labile proton positions. In particular, an indication that back-protonation could be due to amino acid type-specific enzymatic reactions in the cell extract comes from the observations that strong back-protonation at the α-position is observed only for some of the amino acids (Table 2) and that back-protonation at the β-position is even more selective. In designing an exploratory experiment, we hypothesized that racemase activities could be a cause for the amino acid-dependent back-protonation and screened the cell-extract for racemase activities. Thereby we made use of the fact that D-glutamate and D-aspartate are important components of peptidogylcan synthesis in bacterial cell-wall biosynthesis (Sengupta et al. 2006; Veiga et al. 2006), where D-Glu and D-Asp are synthesized from L-Glu and L-Asp, respectively, by specific glutamate- and aspartate-racemases. To make use of the reverse reactions, we added D-Glu and D-Asp instead of their L-counterparts to the cell-free protein synthesis setup, and the amount of protein produced with this variant amino acid mixture was monitored by an immunoassay using anti-His antibodies. These experiments did not yield any significant protein production, showing that there is at most low glutamate- and aspartate-racemase activity, and indicating that other factors than racemase activities in the cell-extract might have a dominant role in the observed back-protonation.

Considering that *E. coli* crude cell extract has a very complex composition, exhaustive elimination of all potential causes for the observed back-protonation might turn out to be a laborious project. As an alternative, one might want to consider the use of the "Pure Translation System" (Shimizu et al. 2005), which is comprised exclusively of components that are required for protein translation. In spite of the as yet low reaction yields of the "Pure Translation System", analytical-scale studies with this system might help to gain further insight into the causes of back-protonation.

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