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ARTICLE

An economic approach to efficient isotope labeling in insect cells using homemade ^{15}N-, ^{13}C- and ^{2}H-labeled yeast extracts

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Abstract Heterologous expression of proteins in insect cells is frequently used for crystallographic structural studies due to the high yields even for challenging proteins requiring the eukaryotic protein processing capabilities of the host. However for NMR studies, the need for isotope labeling poses extreme challenges in eukaryotic hosts. Here, we describe a robust method to achieve uniform protein ^{15}N and ^{13}C labeling of up to 90 % in baculovirus-infected insect cells. The approach is based on the production of labeled yeast extract, which is subsequently supplemented to insect cell growth media. The method also allows deuteration at levels of >60 % without decrease in expression yield. The economic implementation of the labeling procedures into a standard structural biology laboratory environment is described in a step-by-step protocol. Applications are demonstrated for a variety of NMR experiments using the Abelson kinase domain, GFP, and the beta-1 adrenergic receptor as examples. Deuterated expression of the latter provides spectra of very high quality of a eukaryotic G-protein coupled receptor.

Keywords Abelson kinase · GPCR · GFP · Beta-1 adrenergic receptor · Baculovirus · Heteronuclear NMR

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Introduction

Labeling by the stable isotopes ^{15}N, ^{13}C and often also ^{2}H is a prerequisite for the majority of contemporary biomolecular NMR experiments. It is well established for protein expression in the heterologous host *Escherichia coli* where minimal media provide an inexpensive, straightforward manner of isotope incorporation. However, high-quality functional expression of proteins from higher organisms such as humans often requires a higher eukaryotic host system. This seems related to the availability of a proper protein folding, post-translational modification, and membrane insertion machinery, which is less developed or absent in *E. coli*.

The baculovirus/insect cell (*Spodoptera frugiperda*) system is a well-established, highly successful eukaryotic expression system capable of many post-translational modifications, which has been used in numerous crystallographic studies of soluble and membrane proteins. However, the restricted amino acid metabolism of insect cells presents an extreme challenge for isotope labeling. Typical insect cell growth media are complex (O'Reilly et al. 1994) and require the addition of all amino acids in isotope-labeled form. Based on a commercial medium containing labeled amino acids, the uniform ^{15}N and ^{13}C labeling and high-yield expression of the human Abelson kinase domain (c-Abl) has been demonstrated in this system (Strauss et al. 2005). In combination with selective labeling of individual amino acids (Strauss et al. 2003), almost complete backbone assignments and high resolution structural information could be obtained for the 32 kDa c-Abl using this uniformly ^{15}N/^{13}C-labeled material (Vajpai et al. 2008a, b). Although this procedure of uniform ^{15}N and ^{13}C labeling is clearly feasible, it is extremely expensive. Furthermore, for the majority of larger

expression targets, labeling by ^{2}H would be required for optimal sensitivity and resolution. However, the costs of deuterated ^{15}N- and/or ^{13}C-labeled amino acids are prohibitive, and only addition of deuterated, but otherwise unlabeled amino acids has been reported (Kofuku et al. 2014).

As alternative to the addition of labeled amino acids to the insect cell medium the addition of labeled yeast autolysates (yeastolates) has been proposed (Egorova-Zachernyuk et al. 2009). Incorporation of ^{15}N by supplementation of such extracts as the sole amino acid source to an inexpensive dropout medium was demonstrated for the human histamine receptor H1R (Egorova-Zachernyuk et al. 2010). However, the method was not further developed for high-efficiency isotope labeling including ^{13}C and ^{2}H as well as applications to multi-dimensional NMR. A recent refinement of the yeastolate-based approach (Meola et al. 2014) achieved protein expression yields of 6–11 mg/L and ^{15}N incorporation levels between 65 and 80 % by supplementation with a labeled yeast extract from a commercial source of undisclosed composition.

Here we have developed a robust method for the production of ^{15}N- and ^{13}C-labeled yeast extract and its use as supplement in insect cell growth media for protein expression. The method achieves uniform protein ^{15}N and ^{13}C labeling of up to 90 % at typical expression yields of 40 mg/L for soluble proteins. By growing yeast in deuterated media, the procedure can also yield protein deuteration levels of >60 % without decrease in insect cell expression yield. The methods are applied to c-Abl, GFP, and the beta-1 adrenergic receptor. For the latter, ^{2}H,^{15}N labeling provides spectra of so far unprecedented quality for a eukaryotic G-protein coupled receptor. The labeling procedures are described in a step-by-step protocol for economic implementation in a standard structural biology laboratory environment.

Results and discussion

Production and characterization of optimized *Pichia pastoris* yeastolate

Detailed protocols for the yeast fermentation and the production of yeastolate are given in Table 1. In brief, *Pichia pastoris* was grown in a fermenter under fed-batch conditions with typical volumes of 0.5–5 L. Growth on a glucose- and ammonium-based yeast minimal medium (YM) yielded up to 64 g cell wet weight (CWW) of yeast biomass per liter of cell culture medium in ^{15}N- or $^{15}N/^{13}C$-labeled form. Deuteration could be achieved by adapting the yeast cells first on a 70 % D_2O-YM medium, which was followed by a switch to 100 % D_2O-YM. Under these conditions, still 48 g/L CWW were produced, corresponding to a reduction by 25 % relative to the H_2O conditions.

An optimized protocol for *Pichia pastoris* autolysis was developed, which maximizes the amino acid content in the yeast extract. Among others, the protocol contains a zymolyase incubation step to process the yeast cell wall prior to autolysis and papain treatment during the high-temperature incubation phase. The protocol yields on average 6.6 ± 0.7 g lyophilized yeastolate per liter of cell culture, containing 38 ± 4 % (w/w) of free amino acids.

The isotope incorporation levels of the free amino acids in the yeastolates were determined by LC/MS analysis (Supplementary Table S1). Using $^{15}NH_4Cl$ as the sole nitrogen source for yeast growth, an average ^{15}N incorporation of 96 % was observed. Likewise, $^{13}C_6$-glucose as the sole carbon source yielded an average ^{13}C incorporation of 97 %. Growth on 100 % D_2O-YM, but not using any other deuterated ingredients, resulted in an average ^{2}H incorporation of 75 %. This is in good agreement with previously reported deuteration levels in *E. coli* for growth on D_2O and otherwise protonated medium components (Rosen et al. 1996).

Optimization of yeast extract supplement to insect cell medium

To establish the amount of amino acids required as supplement to an amino acid-depleted, yeast extract-free insect cell medium (ΔSF4, Bioconcept), cell proliferation after baculovirus infection as well as the expression of c-Abl were assayed for Sf9 insect cells grown on this medium supplemented by varying amounts of amino acids. Starting with amino acid concentrations according to the established IPL-41 medium (Weiss et al. 1981), the supplementation was step-wise reduced. Reduction to 20 % of the IPL-41 amino acid level did not affect cell viability and protein expression, while any further reduction led to a significant decrease of both parameters.

In a next step, the content of single amino acids in a commercial (Bacto Yeast Extract, Difco) and the *Pichia* yeastolate was quantified by mass spectrometric phenylthiohydantoin amino acid analysis (PTH-AAA). Cysteine was not detectable by PTH-AAA, but an earlier study indicates a low content [<1 % (w/w)] in various yeasts (Martini et al. 1979). Figure 1 shows the determined amino acid concentrations for 8 g/L commercial and *Pichia* yeastolates in comparison to the 20 % IPL-41

Table 1 Fermentation of *Pichia pastoris* and preparation of yeastolate

Preparation of yeast medium (YM)	
Unlabeled YM	1. Prepare unlabeled minimal medium based on glucose and yeast nitrogen base (YNB) in ultrapure water (Milli-Q) as 100 mM potassium phosphate, 2.5 g/L glucose, 2 mg/L biotin, and 6.7 g/L YNB without amino acids (e.g. BD Difco)
	2. Adjust medium to pH 6
	3. Filter-sterilize and store at 4 °C
^{15}N-YM	Follow recipe for unlabeled YM, but use YNB without amino acids and without ammonium (e.g. BD Difco). Add 4.3 g/L $^{15}NH_4Cl$ as nitrogen source
^{13}C,^{15}N-YM	Follow recipe for ^{15}N-YM and replace unlabeled glucose by 2.5 g/L of $^{13}C_6$-glucose
^{2}H,^{15}N-YM and ^{2}H,^{15}N,^{13}C-YM	Prepare ^{15}N-YM or ^{15}N,^{13}C-YM in 99.8 % D_2O. Possibly higher levels of deuteration may be achieved using $^{2}H_7$-glucose or $^{2}H_7$,$^{13}C_6$-glucose respectively. Supply all components as powders to avoid dilution of deuterium
Feeding solution for fed-batch phase	1. Prepare 100 mL of feeding solution per 1 L of fermentation volume based on YM without amino acids and without ammonium, but replace 2.5 g/L by 25 g/L glucose. Use isotope-labeled water and glucose according to your labeling strategy
	2. Adjust solution to pH 6
	3. Filter-sterilize and store at 4 °C
Fermentation of *Pichia pastoris*	
Fermentation in H_2O	1. Grow wild-type *Pichia pastoris* strain (X-33) on fresh LB agar plate at 30 °C
	2. Inoculate unlabeled YM preculture (e.g. 10 mL), grow at 30 °C and 200 rpm (shaking flask) over night to OD_{600} 2
	3. Use preculture to inoculate unlabeled YM scale-up culture (e.g. 100 mL) at OD_{600} 0.2
	4. Grow scale-up culture (shaking flask, 30 °C, 200 rpm) to OD_{600} 2. Harvest cells by centrifugation at 5000*g* for 15 min
	5. Resuspend pelleted cells in labeled YM. Inoculate fermenter to yield a starting OD_{600} of 0.2 in fermentation volume (e.g. 1 L)
	6. Run fermenter-controlled batch-phase at 30 °C, agitation speed 500 rpm, 0.5 L/min airflow, pH 6.0 (adjusted by addition of 4 M NaOH). Maintain dissolved oxygen (DO) level above 35 % by controlled stirring
	7. When initial glucose is fully depleted as judged by increase in DO, start fed-batch phase at a feeding rate to counteract the DO increase
	8. Stop fermentation when fed glucose is depleted. Harvest cells by centrifugation at 5000*g* for 30 min
Fermentation in D_2O	1. Replace unlabeled YM in the scale-up culture with 70 % D_2O-YM for D_2O adaptation
	2. Grow scale-up culture to OD_{600} of 2. Harvest cells by centrifugation at 5000*g* for 15 min
	3. Resuspend cell pellet in 100 % D_2O, labeled YM. Inoculate fermenter to yield a starting OD_{600} of 0.2 in fermentation volume (e.g. 1 L)
	4. Follow H_2O fermentation steps 6–8, but use D_2O feeding and pH control solutions
	5. For recycling of D_2O, supernatants can be sterile-filtered, charcoal-treated, and distilled
Yeastolate preparation	
1. Wash yeast cell pellet with ultrapure water and centrifuge (5000*g*, 30 min)	
2. Prepare a 30 % (w/v) yeast slurry in sterile-filtered water and adjust pH to 7.5 using NaOH	
3. Add 0.5 % (w/w) Zymolyase 20T (Amsbio) and incubate slurry at 35 °C in water bath for 6 h maintaining pH at 7.5	
4. Heat inactivate Zymolyase 20T at 65 °C for 20 min	
5. Adjust to pH 6 and add 0.5 % (w/w) papain powder (Carl Roth)	
6. Incubate slurry under continuous shaking for 5 days at 50 °C	
7. Mechanically homogenize lysate (e.g. using tissue grinder)	
8. Spin down cell debris at 20,000*g* for 30 min and collect supernatant	
9. Filter supernatant through 10 kDa MWCO using a stirred filtration cell (Millipore). Collect flow-through	
10. Lyophilize flow-through to yield a powder of yeastolate. Store at 4 °C	

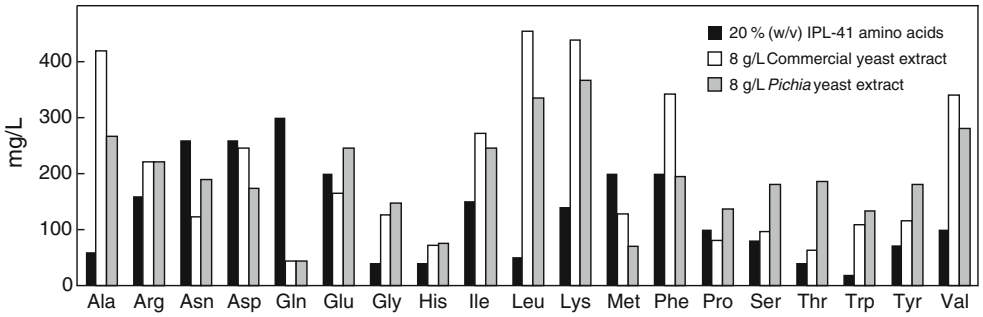

Fig. 1 Amino acid content of different yeast extracts and IPL-41. The free amino acid content of the commercial (Bacto Yeast Extract, Difco, *white*) and *Pichia* yeast extract (*gray*) was quantified by HPLC analysis following pre-column derivatization with phenyl isothiocyanate. Levels of cysteine were not determined by this method. The amino acid content of the yeast extracts is compared to 20 % concentrations of amino acids in the IPL-41 medium (*black*), which was found to be sufficient to promote protein expression in Sf9 insect cells

medium. Interestingly, the overall content of amino acids was comparable in both yeastolates, but differed for individual amino acids. In most cases, the yeastolate amino acid concentrations exceeded those of the 20 % IPL-41 medium. However, glutamine and methionine concentrations were significantly lower in the yeastolates.

The performance of the yeastolate-based insect cell growth media was then assessed by the expression of c-Abl protein in Sf9 cells. Expression in a commercial full medium (Insect Xpress, Lonza) was taken as reference and resulted in yields of 62 mg/L of purified c-Abl (Fig. 2). Following the results of the amino acid analysis, the ΔSF4 medium was initially supplemented by 8 g/L of either commercial or *Pichia* yeastolate without further addition of the low-abundance amino acids glutamine, methionine and cysteine. Under these conditions, expression levels dropped to only 2.4 mg/L purified c-Abl. Hence, the ΔSF4 was further supplemented by 1.0 g/L glutamine and 1.0 g/L methionine (20 % IPL-41). This dramatically increased the yield to 45 mg/L of purified c-Abl, recovering 73 % of the full medium expression. A subsequent test showed that the additional supplementation of methionine was not required, since its omission did not alter the expression level (Fig. 2). Also no further increase was observed upon supplementation of 0.23 g/L cysteine (20 % IPL-41), indicating that addition of cysteine was not necessary. This observation may be explained by previous findings that Sf9 cells can synthesize cysteine from methionine (Doverskog et al. 1998). It has been reported that Sf9 cells are able to synthesize glutamine from ammonium (Drews et al. 2000) and thus glutamine may be substituted by

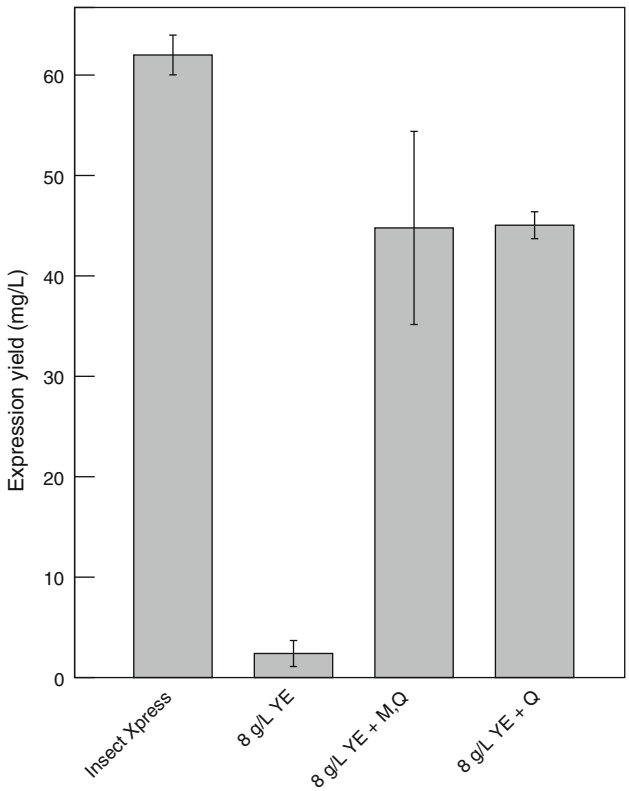

Fig. 2 Expression yield of c-Abl in Sf9 insect cells under various conditions. A reference yield was obtained in the commercial Insect Xpress medium (Lonza). Different supplements to SF4 medium depleted in amino acids and yeastolate (ΔSF4) were tested: 8 g/L of *Pichia* yeastolate (YE), 8 g/L YE + 1 g/L methionine + 1 g/L glutamine (YE + M, Q), and 8 g/l YE + 1 g/L glutamine (YE + Q). Cell cultures in supplemented ΔSF4 were starved for 2 h prior to final medium exchange. All yields were quantified by UV absorption of purified protein at 280 nm

ammonium in Sf9 expression cultures (Meola et al. 2014). However, the substitution by up to 10 mM ammonium resulted in expression levels that were below 10 % of the glutamine-supplemented cultures. Therefore, ΔSF4 supplemented by 8.0 g/L YE and 1.0 g/L glutamine was taken as the optimal medium for all further experiments.

Expression of ^{15}N-labeled c-Abl and trGFPuv

Based on this optimized medium ^{15}N-labeled c-Abl was produced for NMR analysis. Initially, insect cells were grown in unlabeled medium and then changed to the medium containing ^{15}N-yeastolate and $^{15}N_2$-glutamine immediately prior to virus infection. This resulted in yields of 40 mg/L purified protein and an average ^{15}N incorporation of 77 % as determined by mass spectrometry. Since spillover from the initial unlabeled medium was suspected as the source of the low labeling, a starvation step in ΔSF4 without yeastolate and glutamine supplement was introduced 2 h prior to the exchange to ΔSF4 medium with labeled yeastolate and glutamine. The starvation step increased the ^{15}N incorporation to 89 % without reduction of the expression level (Table 3). A similarly high ^{15}N incorporation of 90 % (Table 3) was obtained for the expression of a truncated (Met1-Ile229), optimized form of green fluorescent protein (trGFPuv) (Khan et al. 2003) with a yield of 40 mg/L. Hence, all subsequent expressions were performed with the 2 h starvation step included in the labeling protocol (Table 2).

Figure 3 shows an ^{1}H-^{15}N HSQC spectrum recorded on the ^{15}N-labeled c-Abl protein expressed in insect cells grown on the ^{15}N-yeastolate. The resonances are identical to previously published spectra of c-Abl (Vajpai et al. 2008b). The HSQC resonance intensities were compared quantitatively to an HSQC recorded under identical conditions on a ^{15}N-labeled c-Abl sample prepared from insect cells grown on the commercial, uniformly ^{15}N-labeled BioExpress-2000 (CIL #CGM-2000-N) medium (Strauss et al. 2005; Vajpai et al. 2008b). For the latter, an average ^{15}N labeling efficiency of 91.4 % based on mass spectrometry has been reported. After correction for the respective concentrations, the resonance intensities agreed within ~10 % for all amino acid types (data not shown). This corroborates the very similar ^{15}N incorporation obtained by mass spectrometry and indicates that there is no obvious specific ^{15}N incorporation for certain amino acids.

Table 2 Expression of labeled proteins in insect cells based on yeastolate

Preparation of labeling medium	
Prepare stock solution of yeastolate (e.g. 200 g/L) in sterile ultrapure water. Filter-sterilize and store aliquots at −20 °C	
^{15}N labeling	Supplement dropout medium depleted of amino acids and yeast extract (e.g. ΔSF4, BioConcept) with 8 g/L ^{15}N-yeastolate and 1 g/L $^{15}N_2$-glutamine
^{15}N,^{13}C labeling	Supplement dropout medium depleted of amino acids, yeast extract and all carbon sources (e.g. ΔSF4, BioConcept) with 8 g/L ^{15}N,^{13}C-yeastolate, 5 g/L $^{13}C_6$-glucose and 1 g/L $^{15}N_2$-glutamine
^{2}H,^{15}N,^{13}C labeling	As ^{15}N,^{13}C labeling but use 8 g/L ^{2}H,^{15}N,^{13}C-yeastolate
Adjust pH to 6.2 (±0.2)	
Filter-sterilize medium and store at 4 °C	
Labeled expression in insect cells	
1. Adapt Sf9 or Sf21 cells to growth in serum-free medium (e.g. Insect Xpress, SF4) and optimize expression conditions at 27 °C, shaken at 80 rpm in Erlenmeyer flasks	
2. Scale up cells in mid-log phase of growth to desired expression volume (typically $1.5 \times 10^6 - 2 \times 10^6$ cells/mL)	
3. When the required cell density is reached, harvest cells by centrifugation at 200*g* for 4 min (20 °C)	
4. Resuspend cells in dropout medium of same volume (depleted of amino acids and yeast extract) and starve cells for 2 h (27 °C, 80 rpm)	
5. Determine cell density and viability. Spin down amount of cells required for expression (300*g*, 4 min)	
6. Resuspend cells in labeling medium with half the volume of the final culture	
7. Infect with baculovirus according to optimized conditions (MOI, functional titer). Avoid label dilution by using high-titer stocks	
8. Adjust to final culture volume after 6 h	
9. Grow baculovirus-infected cells for 48–72 h in labeled medium (27 °C, 80 rpm)	
10. Harvest cells at 1000*g* for 10 min and purify recombinant protein	

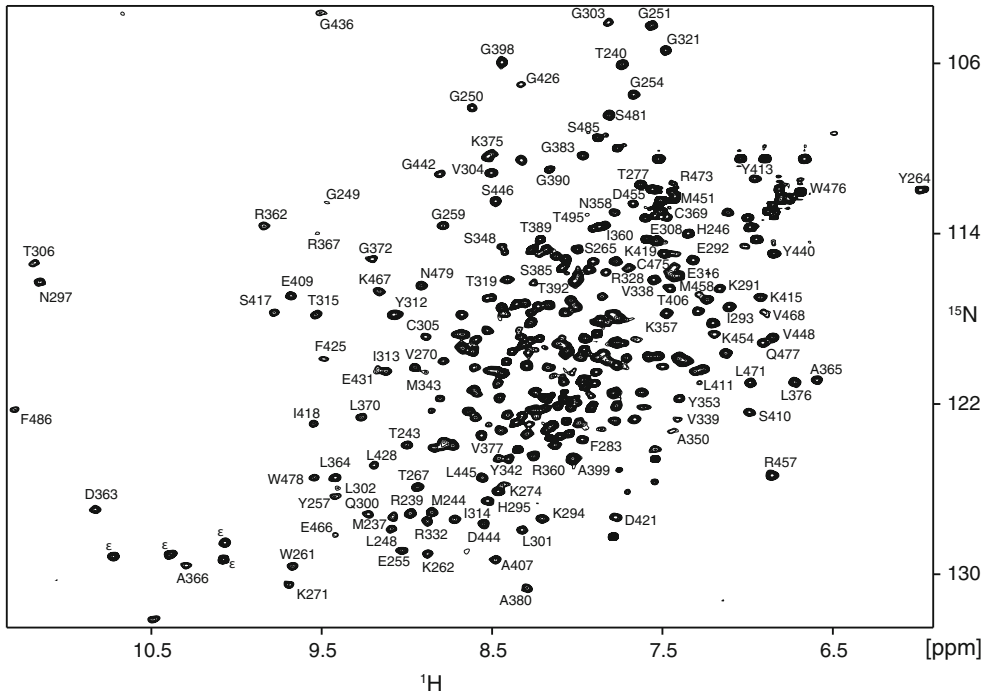

Fig. 3 ^{1}H-^{15}N HSQC spectrum of ^{15}N-labeled c-Abl expressed in Sf9 insect cells. The protein was expressed in Sf9 cells grown on ^{15}N-yeastolate and $^{15}N_2$-glutamine as described in the text. The spectrum was recorded in 2 h on a 200 μM ^{15}N-labeled c-Abl sample

Expression of ^{15}N,^{13}C-labeled trGFPuv and triple resonance NMR

To test its usefulness for canonical triple resonance protein NMR experiments, an ^{1}H,^{15}N,^{13}C-labeled sample of trGFPuv was expressed according to the protocol given in Table 2 using ^{15}N,^{13}C-yeastolate and resulting in a yield of 39 mg/L. The labeling procedure is similar as for the ^{15}N labeling. However, to achieve optimal ^{13}C labeling, the ΔSF4 medium was further depleted of glucose, maltose and sucrose, and 5 g/L of $^{13}C_6$-glucose were supplemented as the only sugar source. Glutamine was only supplemented in

Table 3 Isotope incorporation of proteins expressed using different yeastolate labeling schemes

Protein	Label	Observed mass (Da)	Observed shift (Da)[a]	Expected shift (Da)[b]	Incorporation ratio[c]
c-Abl	Unlabeled	34,892.6[d]	–	–	–
	^{15}N	35,249.2	356.6	402	0.89
	^{15}N,^{13}C	36,558.7	1666.1	1934	0.85[e]
	^{2}H,^{15}N	36,337.4	1444.8	2236	0.61[f]
trGFPuv	Unlabeled	25,585.3	–	–	–
	^{15}N	25,859.6	274.3	305	0.90
	^{15}N,^{13}C	26,778.0	1192.7	1409	0.84[g]

[a] Calculated as the difference between observed mass and unlabeled reference

[b] Theoretical mass shift based on total number of atoms to be labeled

[c] Ratio of the observed by the expected mass shift

[d] The N-terminus of c-Abl kinase domain starts with S*YYHHHHHHDYDIPTTENLYFQGAMDP-c-Abl(S^{229}–S^{500}), * N-acetylation of serine (Strauss et al. 2003)

[e] ^{13}C incorporation assuming 1532 labeled carbon atoms and 0.89 ^{15}N incorporation (356.6 Da)

[f] ^{2}H incorporation assuming 1789 non-exchangable protons and 0.89 ^{15}N incorporation (356.6 Da)

[g] ^{13}C incorporation assuming 1099 labeled carbon atoms and 0.90 ^{15}N incorporation (274.3 Da)

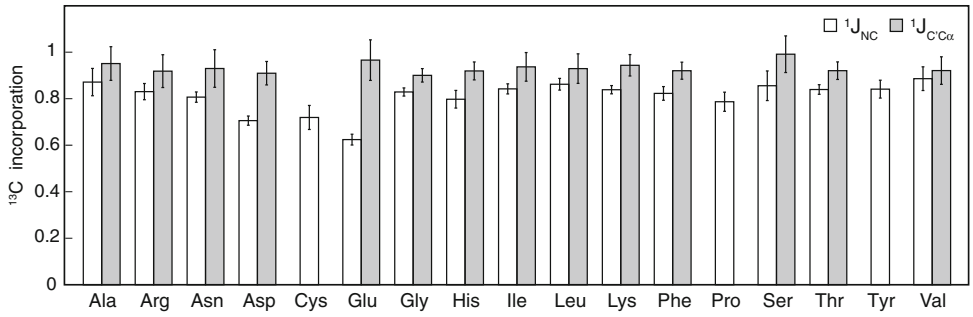

Fig. 4 Specific ^{13}C incorporation in $^{15}N,^{13}C$-GFP analyzed by NMR spectroscopy. $^{15}N,^{13}C$-GFP was expressed in Sf9 cells grown on $^{15}N,^{13}C$-yeastolate and $^{15}N_2$-glutamine. ^{13}C enrichments of the carbonyl nucleus preceding a ^{15}N amide determined via the $^{1}J_{NC'}$ scalar coupling (*white*) and of a C^{α} nucleus next to a $^{13}C'$ carbonyl determined via the $^{1}J_{C'C\alpha}$ scalar coupling (*gray*) are shown as averages and standard errors for the different amino acid types that could be detected. Details are given in Supplementary Figures S1 and S2

^{15}N-labeled form, since addition of the considerably more expensive ^{15}N, ^{13}C-glutamine did not result in a significant increase of ^{13}C labeling as judged by mass spectrometric analysis. An overall ^{13}C incorporation of 84 % was estimated by mass spectrometry, subtracting the previously determined 90 % ^{15}N incorporation from the total mass (Table 3).

The efficiency of ^{13}C incorporation into specific atom positions was further analyzed by NMR spectroscopy. Using modulation by the one-bond $^{1}J_{NC'}$ scalar coupling in the ^{15}N evolution period of a constant-time ^{1}H-^{15}N HSQC (Supplementary Figure S1), the ^{13}C enrichment of the carbonyl nucleus of the amino acid preceding the ^{1}H-^{15}N amide could be determined. Figure 4 shows averages and standard deviations of this enrichment according to the different observed amino acid types. The average ^{13}C incorporation determined by this experiment amounts to 81 %, which is slightly lower than the 84 % determined by mass spectrometry. This reduction may be explained by systematic errors in the NMR determination due the imperfection of the $^{13}C'$ coupling pulse and the faster relaxation of the ^{15}N-{$^{13}C'$} antiphase term, which diminishes the apparent size of the ^{13}C signal (Grzesiek et al. 1993). The amino acid-specific analysis reveals that most amino acids have higher than 80 % (uncorrected NMR value) ^{13}C incorporation at the carbonyl position. However, incorporation is reduced for glutamate (62.4 %), aspartate (71 %), and cysteine (72 %). This is consistent with the reported metabolic conversion of (^{12}C-)glutamine into the latter amino acids in insect cells (Drews et al. 2000).

The 81 % ^{13}C incorporation determined from the one-bond $^{1}J_{NC'}$ scalar coupling corresponds to the probability for finding a $^{13}C'$ nucleus in the amino acid preceding an ^{15}N-labeled amide. However, within a single labeled amino acid the probability for a next neighbor ^{13}C carbon should be higher due to the overall 96 % ^{13}C content of the amino acids coming from the yeastolate (Supplementary Table S1). This was tested by measuring the incorporation of $^{13}C^{\alpha}$ nuclei next to $^{13}C'$ nuclei in a modified HNCO, which was modulated by the one-bond $^{1}J_{C'C\alpha}$ coupling in a constant-time $^{13}C'$ evolution period (Supplementary Figure S2). These intraresidue next neighbor ^{13}C incorporation ratios are also presented in Fig. 4 according to the different amino acid types. No pronounced variations are observed. The average of the intraresidue next neighbor ^{13}C incorporation within a labeled amino acid amounts to 93 % and is clearly considerably higher than the sequential incorporation. Again, the ^{13}C incorporation derived by the NMR experiment presents a lower estimate due to imperfections of the $^{13}C^{\alpha}$ coupling pulse and the faster relaxation of the $^{13}C'$-{$^{13}C^{\alpha}$} antiphase term. The high level of intraresidue next neighbor ^{13}C incorporation agrees with the expected low metabolic conversion of amino acids and has the practical advantage that NMR experiments, which rely on intraresidue heteronuclear connectivities, do not suffer from potentiated losses in sensitivity due to low next neighbor labeling.

The capability to achieve sequential assignments was demonstrated by recording HNCO and HNCA spectra on the $^{15}N,^{13}C$-labeled trGFPuv. Figure 5 shows sequential strip plots from the 3D experiments. Overall, 97 % (89 %) of all expected crosspeaks were observed for the HNCO (HNCA) using an experimental time of 17 h (69 h) on a 230 μM sample of this 26 kDa protein.

Expression of $^{2}H,^{15}N$-labeled c-Abl

To test deuterium labeling, $^{2}H,^{15}N$-labeled c-Abl was expressed in insect cells grown on ΔSF4 medium supplemented by 1 g/L $^{15}N_2$-glutamine and 8 g/L $^{2}H,^{15}N$-labeled

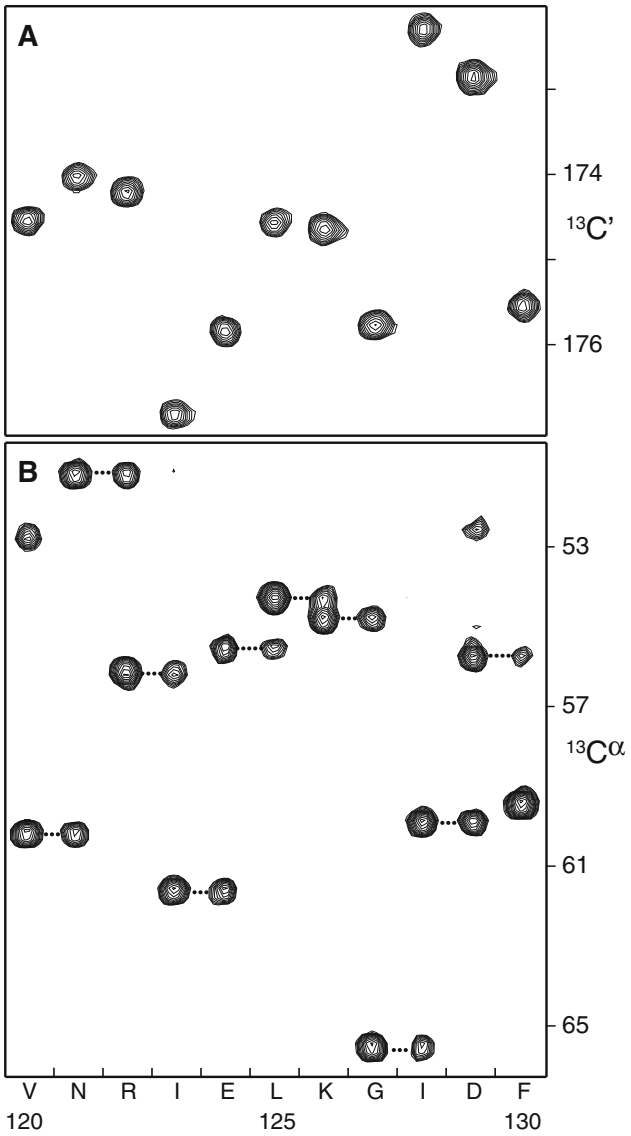

Fig. 5 Triple resonance backbone correlation experiments recorded on ^{15}N,^{13}C-GFP. Strip plots from the 3D data sets of HNCO (**a**) and HNCA (**b**) experiments are displayed for a consecutive stretch of amino acids. ^{15}N,^{13}C-GFP was expressed in Sf9 cells grown on ^{15}N,^{13}C-yeastolate and $^{15}N_2$-glutamine

yeastolate, which had been obtained from a D_2O/$^{15}NH_4Cl$ yeast culture with otherwise protonated medium compounds (Table 1). A yield of 43 mg/L purified ^{2}H,^{15}N-c-Abl was achieved, which is identical to the ^{15}N-labeled or ^{15}N,^{13}C-labeled expression. Mass spectroscopic analysis indicated an average ^{2}H incorporation of 61 % (Table 3), which corresponds to a 19 % drop from the 75 % initial deuteration level of the yeastolate amino acids (Supplementary Table S1).

The benefits of the deuterium labeling were evident in an initial NMR analysis of average amide proton T_2 values, which increased from 10.8 ms in ^{1}H,^{15}N-labeled to 16.2 ms in ^{2}H,^{15}N-labeled c-Abl. An example for the improvement in the spectral sensitivity is given in Fig. 6 for the case of NOESY experiments. Figure 6 shows the amide regions of ^{1}H,^{1}H-NOESY spectra recorded under identical conditions with a mixing time of 230 ms on protonated (Fig. 6a) and deuterated (Fig. 6b) c-Abl. Clearly many more cross peaks become visible for the deuterated case. Furthermore, ^{15}N-edited 3D NOESY spectra were recorded with optimized mixing times of 100 and 230 ms, corresponding to the decay times of the diagonal peaks (selective T_1), for the protonated and deuterated c-Abl, respectively. Obviously, the sensitivity is tremendously increased for the deuterated c-Abl sample (Fig. 6d) and many i, i + 1 and i, i + 2 connectivities are detected, which are not present for protonated c-Abl (Fig. 6c).

Expression of ^{2}H,^{15}N-labeled beta-1 adrenergic receptor

To apply the yeastolate labeling procedure to a highly challenging protein, ^{2}H, ^{15}N-labeled thermostabilized beta-1 adrenergic receptor (β_1AR) (Warne et al. 2008) was expressed in Sf9 cells as described before. A yield of 0.8 mg/L purified, detergent-solubilized protein was achieved, which was comparable to yields from the commercial Insect Xpress (Lonza) medium. The fractional deuteration increased the amide proton T_2 from 3.9 ms observed for the protonated receptor to 5.9 ms for the ^{2}H,^{15}N-labeled receptor. Likewise, the ^{15}N TROSY T_2 increased from 21 to 29 ms upon deuteration. Figure 7 shows the well-resolved TROSY-HSQC recorded for 13 h on the 85 μM ^{2}H,^{15}N-labeled receptor dissolved in a decyl-maltoside detergent micelle (total molecular weight ~100 kDa). About 240 of the 304 expected backbone amide resonances are observable.

Cost analysis

An estimate of the total cost of various isotope labeling schemes in insect cells based on the described YE is provided in Table 4 using current market prices for labeled ingredients and insect cell media. The estimated cost of 1 L insect cell medium for pure ^{15}N labeling amounts to 970 €, which is slightly higher than the estimate of 800 € given for a commercial source of YE-based medium (Meola et al. 2014). However, it should be noted that by the present

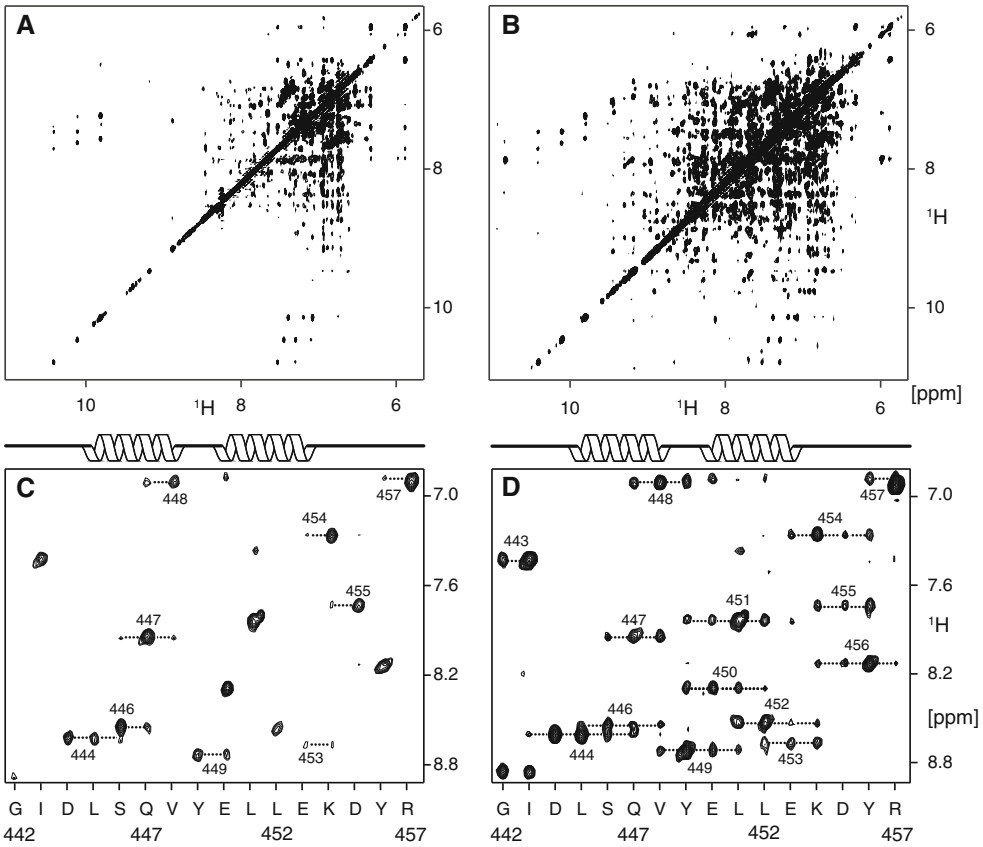

Fig. 6 NOE sensitivity enhancement by deuteration. *Top*: 2D ^{1}H-^{1}H-NOESY spectra recorded under identical conditions on 200 µM samples of ^{15}N-c-Abl (**a**) and ^{2}H,^{15}N-c-Abl (**b**) expressed in Sf9 insect cells (NOE mixing time of 230 ms). *Bottom*: strip plots for a consecutive stretch of amino acids from 3D ^{15}N-edited NOESYs recorded on the same ^{15}N-c-Abl (**c**) and ^{2}H,^{15}N-c-Abl (**d**) samples under identical conditions with the exception of optimized NOE mixing times of 100 ms (^{15}N-c-Abl) and 230 ms (^{2}H,^{15}N-c-Abl)

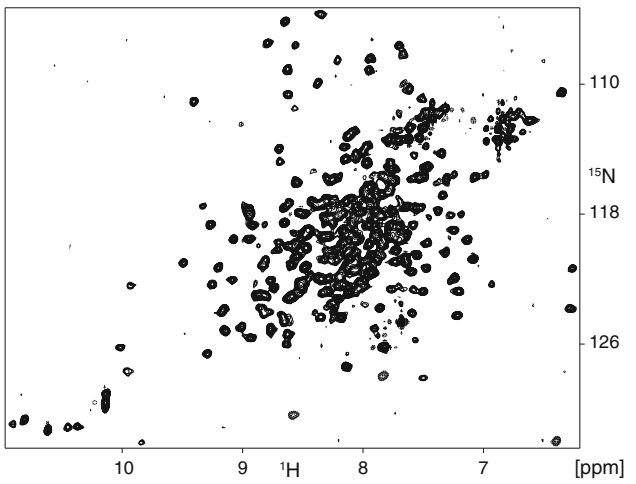

Fig. 7 ^{1}H,^{15}N-TROSY spectrum of ^{2}H,^{15}N-labeled beta-1 adrenergic receptor. ^{2}H,^{15}N-labeled β_1AR was expressed in Sf9 cells grown on ^{2}H,^{15}N-yeastolate and $^{15}N_2$-glutamine as described in the text. The TROSY was recorded in 13 h on 85 µM ^{2}H,^{15}N-labeled β_1AR solubilized in *n*-decyl-β-D-maltopyranoside

method about 4 times higher yields (40 mg/L protein) and 90 % labeling have been achieved without additional costs from a purge phase of insect cell growth on labeled medium. The high expression yield translates into a cost of only 63 € for a typical NMR sample of 0.3 mM Abl kinase domain (MWT 32 kDa) in a 270 µL volume. Furthermore, the largest part of the ^{15}N-labeled medium costs stems from the addition of 1 g/L of ^{15}N-labeled glutamine (800 €). This expense may be reduced to negligible levels by enzymatic synthesis of $^{15}N_2$-glutamine via ^{15}N-glutamate, which can be produced by glutamate dehydrogenase from ^{15}N-labeled ammonium and 2-oxoglutaric acid (Kragl et al. 1993) and subsequently be converted to $^{15}N_2$-glutamine by glutamine synthetase under the addition of ^{15}N-labeled ammonium (Hansen et al. 1992).

Table 4 indicates that compared to ^{15}N labeling, the additional cost for partial deuteration (>60 %) is relatively small (1398 € for 1 L ^{2}H,^{15}N insect cell medium). Since the costs arise from the D_2O used for yeast growth, they may again be reduced considerably by recycling D_2O via distillation. Due to the high amount of glucose required for the yeast growth (27.5 g/L), costs for YE-based insect cell medium increase significantly for ^{13}C labeling and amount to 4424 € for 1 L of ^{15}N,^{13}C insect cell medium. Again the additional cost for the fractional deuteration is relatively minor (4848 € for 1 L ^{2}H,^{15}N,^{13}C insect cell medium). The estimated costs for one sample of triple-labeled Abl kinase domain are still only 314 € under these conditions.

Table 4 Cost estimates for yeastolate-based isotope labeling in insect cells[a]

1 L yeast medium				
Labeling	$^{15}NH_4Cl$[b]	$^{13}C_6$-glucose[c]	D_2O[d]	Total
^{15}N	86	–	–	86
$^{15}N,^{2}H$	86	–	350	436
$^{15}N,^{13}C$	86	2475	–	2561
$^{15}N,^{13}C,^{2}H$	86	2475	350	2911

1 L insect cell medium				
Labeling	Yeastolate[e]	Glucose[f]	Total[g]	c-Abl sample costs[h]
^{15}N	104	–	974	63
$^{15}N,^{2}H$	528	–	1398	91
$^{15}N,^{13}C$	3104	450	4424	287
$^{15}N,^{13}C,^{2}H$	3528	450	4848	314

[a] All costs are given in Euro using conservative estimates of market prices for labeled ingredients and growth media

[b] 4.3 g/L $^{15}NH_4Cl$

[c] 27.5 g/L $^{13}C_6$-glucose

[d] 1 L D_2O

[e] Costs for 8 g isotope-labeled yeastolate based on a yield of 6.6 g per 1 L yeast culture

[f] Addition of 5 g/L $^{13}C_6$-glucose for ^{13}C labeling

[g] Total costs of yeastolate, glucose and 1 g $^{15}N_2$-glutamine (800 EUR) supplements to 1 L of insect cell culture dropout medium (70 EUR, SF4, Bioconcept)

[h] Costs for a 270-µL, 300-µM c-Abl (32 kDa) sample based on an expression yield of 40 mg/L

Conclusion

We have developed a robust protocol for stable isotope labeling of proteins in insect cells based on labeled yeastolate. Our approach overcomes low incorporation rates and low yields described in previous yeastolate-based labeling approaches. This was achieved by a quantitative analysis of the yeastolate amino acid content, supplementation of lacking glutamine, site-specific quantification of isotope incorporation and subsequent optimization. In particular, significant dilution of the isotope labels was prevented by introducing an initial starvation step. In addition to ^{15}N labeling, also efficient $^{15}N,^{13}C$ and partial (>60 %) ^{2}H labeling is demonstrated. Higher ^{2}H incorporation may be achievable by growing yeast on deuterated glucose in addition to D_2O. The described uniform deuteration via the yeastolate may also be combined with the supplementation of specifically labeled amino acids for further gains in resolution and sensitivity (Kofuku et al. 2014).

The preparation of the labeled yeastolate can be readily implemented in a standard, academic structural biology laboratory and does not rely on commercial media of undisclosed content. The costs are moderate for ^{15}N and fractional ^{2}H labeling and may be reduced further by synthesis of labeled glutamine. Labeling by ^{13}C increases the costs, but due to the high yields, still allows production of protonated or partially deuterated $^{15}N,^{13}C$-samples of 'difficult' eukaryotic proteins such as c-Abl at moderate costs of several hundred Euros.

It is hoped that the proposed method will enable detailed studies of eukaryotic proteins such as G-protein-coupled receptors that so far have been inaccessible to NMR due to their requirement of expression in a eukaryotic host. As initial example we provide a well-resolved spectrum of the $^{2}H,^{15}N$-labeled beta-1 adrenergic receptor. Besides their application for insect cells, the described homemade yeast extracts may also serve as a basis for isotope labeling in other higher eukaryotic expression systems such as Chinese hamster ovary and human embryonic kidney cells (Egorova-Zachernyuk et al. 2009).

Materials and methods

Yeast growth and preparation of yeast extract

Detailed protocols are presented in Table 1.

Quantitative amino acid analysis by LC/MS

Crude yeast extract was purified by solid phase extraction using a strong cation-exchange matrix (DSC-SCX SPE, Sigma). Briefly, lyophilized powder was dissolved in 30 mM phosphoric acid solution (pH 2.7) and applied to the conditioned SCX column. The packed column was washed two times with 30 mM phosphoric acid solution. Samples were eluted with 2.5 M NH_4OH containing 10 % acetonitrile and dried in a centrifugal evaporator.

Purified powder was dissolved in 50 % methanol to yield a 10 µg/µL solution for pre-column derivatization with phenyl isothiocyanate (PITC). Two volumes of a freshly prepared coupling solution containing methanol/triethylamine/PITC (7:2:1) were added and the solution was derivatized for 30 min at room temperature protected from light. After derivatization, the mixture was dried under vacuum. Unlabeled standards for each amino acid were prepared accordingly.

The derivatized phenylthiohydantoin (PTH) amino acids were taken up in 20 % acetonitrile for LC/MS analysis. Optimal separation was achieved by HPLC (Agilent) on an Eclipse XDB-C18 reverse phase column (Agilent) at a flow rate of 2.1 mL/min and 50 °C using a gradient elution of 50 mM ammonium acetate at pH 7.2 (Eluent A) against 46:44:10 100 mM ammonium acetate (pH 7.2)/acetonitrile/methanol (Eluent B) (Supplementary Table S2). The PTH

amino acids were detected by optical absorption at 254 and 280 nm, and subsequently identified and quantified for their isotope content on a Bruker microTOF electrospray ionization mass spectrometer. The amount of individual amino acids was determined from the integration of the absorbance peak area and referencing to the unlabeled standard.

Determination of isotope incorporation of expressed proteins by MS

Purified protein samples were desalted by solid phase extraction on C4 reverse phase micro spin columns according to the supplier's protocol (The Nest Group). Isotope incorporation was determined by ESI-TOF mass spectrometry (Bruker microTOF) applying direct infusion of the prepared proteins. Mass spectra were analyzed by maximum entropy deconvolution.

Cell culture and preparation of recombinant baculovirus

In general, handling and maintenance of Sf9 cell culture was based on established protocols (O'Reilly et al. 1994). Permanent cultures were subcultured in mid-log phase and kept in full SF4 medium (Bioconcept) and Insect Xpress medium (Lonza) at 27 °C and shaken at 80 rpm. Recombinant bacmid DNA was generated in DH10Bac *E. coli* cells using the commercial pFastBac vector according to the supplier's procedure (Life Technologies). Baculovirus was generated in adherent cultures as reported by O'Reilly et al. (O'Reilly et al. 1994). Subsequently, high-titer virus stocks for expression were produced by two additional amplification rounds in suspension cultures. In the first amplification round, 50 mL of Sf9 cell culture containing 1×10^6 -cells/mL in serum-free medium were infected with recombinant virus and cultured until cell viability decreased to $\leq$90 %. The supernatant (P1) was collected by centrifugation at 1000*g* and 4 °C for 15 min. For the second amplification round, 100 mL of serum-free Sf9 cell culture containing 1×10^6 cells/mL was infected with P1 and processed accordingly to yield a P2 virus stock.

Functional titration of the P2 virus stock was applied to determine the optimal amount for expression. Briefly, small-scale cultures of Sf9 cells were infected in full SF4 medium at a cell density of 1.5×10^6 cells/mL with P2 virus stock corresponding to functional titers of 1–10 mL per 1 L of cell culture. The yield of purified protein was then quantified for different expression times.

Expression and purification of Abelson kinase domain in insect cells

Samples of Abelson kinase domain (c-Abl) in complex with imatinib were prepared according to the previously published protocol leaving out the TEV cleavage step of the N-terminal polyhistidine tag (Vajpai et al. 2008a). NMR samples were prepared in 20 mM BIS–TRIS (pH 6.5), 150 mM NaCl, 2.5 mM TCEP, 5 % D_2O and 0.02 % NaN_3.

Cloning and purification of truncated trGFPuv

A synthesized gene sequence of the truncated GFP cycle 3 mutant (trGFPuv) based on the previously published construct (Huang et al. 2007) was purchased from a commercial supplier (Genewiz). The sequence was codon-optimized for expression in insect cells. Recombinant baculovirus was prepared by insertion of the trGPFuv gene into the pFastBac vector (Life Technologies). Bacmid DNA was produced in DH10Bac *E. coli* cells according to the standard protocol provided by the supplier.

Functional virus titer and the optimal expression time were determined in small-scale expression tests. trGFPuv was subsequently expressed in SF4-adapted Sf9 insect cells until the cell viability decreased to 90 %. Cells were harvested at 1000*g* for 10 min and lysed by sonication. The protein was purified following the established protocol in *E. coli* (Huang et al. 2007). NMR samples were prepared in 20 mM sodium phosphate (pH 7.4), 5 % D_2O and 0.02 % NaN_3.

Expression and purification of turkey beta-1 adrenergic receptor

Purification of the thermostabilized, turkey beta-1 adrenergic receptor [mutant JM3 (Miller and Tate 2011)] was adapted from the established protocol (Warne et al. 2008). Since the receptor was prepared for solution NMR, solubilization and subsequent purification steps were carried out in *n*-decyl-β-D-maltopyranoside (DM). NMR samples were prepared in 20 mM TRIS/HCl (pH 7.5), 100 mM NaCl, 1 mM alprenolol hydrochloride (racemic), 5 % D_2O, 0.1 % DM and 0.02 % NaN_3.

NMR spectroscopy

NMR experiments were recorded on a Bruker Avance III 900 MHz spectrometer equipped with a triple-resonance cryoprobe. Amide proton T_2 values were determined from

a jump-return spin-echo experiment (Sklenar and Bax 1987). Amide ^{15}N T_2 values were determined from the decay of a ^{15}N spin echo in a TROSY experiment.

All experiments on c-Abl were acquired at a temperature of 25 °C. ^{1}H-^{15}N HSQC experiments on ^{15}N-c-Abl were recorded with 75 (^{15}N) × 1024 (^{1}H) complex points and acquisition times of 30 ms (^{15}N) and 40 ms (^{1}H). 2D ^{1}H,^{1}H-NOESY experiments on ^{15}N-c-Abl and ^{2}H,^{15}N-c-Abl were recorded with 388 (^{1}H) × 2048 (^{1}H) complex points, acquisition times of 18.6 ms (^{1}H) × 80 (^{1}H) ms, and a total experimental time of 6.5 h. The 3D ^{15}N-NOESY-HSQC experiments were recorded with 268 (^{1}H) × 130 (^{15}N) × 2048 (^{1}H) complex points, acquisition times of 16 ms (^{1}H) × 30 ms (^{15}N) × 80 (^{1}H) ms, and a total experimental time of 77 h.

All experiments on trGFPuv were acquired at a temperature of 37 °C. The 3D HNCO experiment on ^{15}N,^{13}C-trGFPuv was recorded with 104 (^{15}N) × 100 (^{13}C) × 2048 (^{1}H) complex points, acquisition times of 27 ms (^{15}N) × 20 ms (^{13}C) × 80 (^{1}H) ms, and a total experimental time of 17 h. The 3D HNCA experiment on ^{15}N,^{13}C-trGFPuv was recorded with 86 (^{15}N) × 82 (^{13}C) × 2048 (^{1}H) complex points, acquisition times of 22 ms (^{15}N) × 9 ms (^{13}C) × 80 (^{1}H) ms, and a total experimental time of 69 h.

The 2D ^{1}H,^{15}N-TROSY experiment on ^{2}H,^{15}N-β_1AR was recorded at 30 °C with 150 (^{15}N) × 1024 (^{1}H) complex points, acquisition times of 30 ms (^{15}N) × 43 (^{1}H) ms, and a total experimental time of 13 h.

Data processing and analysis

All NMR data were processed with the NMRPipe software package (Delaglio et al. 1995). Protein assignments and analysis of peak intensities were carried out using the programs Sparky (Goddard and Kneller 2008) and PIPP (Garrett et al. 1991).

Quantification of ^{13}C incorporation by NMR

Details of the constant-time ^{1}H-^{15}N HSQC and HNCO used for the quantitative assessment of ^{13}C incorporation at the carbonyl and C^{α} position are given in Supplementary Figures S1 and S2.

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