Journal of Biomolecular NMR, **26:** 367–372, 2003.
KLUWER/ESCOM

Amino–acid-type selective isotope labeling of proteins expressed in Baculovirus-infected insect cells useful for NMR studies

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Received 11 December 2002; Accepted 17 March 2003

Key words: Abl kinase, Baculovirus, HSQC, NMR, selective labeling, STI-571

Abstract

Culture conditions for successful amino–acid-type selective isotope labeling of proteins expressed in Baculovirus-infected insect cells are described. The method was applied to the selective labeling of the catalytic domain of c-Abl kinase with ^{15}N-phenylalanine, ^{15}N-glycine, ^{15}N-tyrosine or ^{15}N-valine. For the essential amino acids phenylalanine, tyrosine and valine high ^{15}N-label incorporation rates of $\geq$90% and approximately the expected number of resonances in the HSQC spectra were observed, which was not the case for the non-essential amino acid glycine. The method should be applicable to amino-acid-type selective isotope labeling of other recombinant proteins which have not been amenable to NMR analysis.

The availability of sufficient amounts of high-quality protein is often the bottleneck for structural studies by x-ray crystallography or NMR. For the production of isotopically (^{13}C, ^{15}N, and/or ^{2}H) labeled proteins, recombinant proteins are typically expressed in *E. coli*. A variety of methods is available to produce isotopically labeled proteins in this organism, including methods for full labeling, backbone-only labeling, methyl group labeling, amino–acid-type selective labeling, segmental labeling and 'reverse' labeling (reviewed by Goto and Kay, 2000; Lian and Middleton, 2001). However many proteins of interest to pharmaceutical research are not readily expressed in *E. coli*. Problems with expression of good quality proteins in *E. coli* may be caused by insolubility of the protein or inability of its posttranslational modification. If expression in *E. coli* is not feasible, other expression systems such as *Pichia pastoris*, CHO cells, HEK cells, or Baculovirus-infected insect cells can be used for producing proteins for structural studies (Goto and Kay, 2000). Alternatively, cell-free expression or chemical synthesis may be attempted. Proteins produced by these methods have been crystallized. However, isotopically labeling proteins in expression systems with animal cell cultures is more difficult and more expensive. This is due to the need to use well-defined expression media supplemented with expensive labeled amino acids leading to reduction in expression levels. Amino-acid-type selective isotope (aasi) labeling, which is a well-established technique for *E. coli* host cells (Muchmore et al., 1989), has been described for CHO cells (Archer et al., 1993; Lustbader et al., 1996). For aasi-labeling of rec. proteins expressed with Baculovirus-infected insect cells, no successful protocol giving satisfactory label incorporation rates has been published so far. Incorporation of L-(ring-$^{2}H_4$)Tyrosine with 70% (de Lange et al., 1998) and (α, ε-$^{15}N_2$)Lysine with 60% incorporation rate (Creemers et al., 1999) into rhodopsin expressed in Baculovirus-infected Sf9 cells has been reported. In addition, a method for selenomethionine-labeling proteins expressed with Baculovirus-infected insect cells was presented by Bellizzi et al. (1999).

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In our experience, Baculovirus expression is very valuable and often successful in cases where *E. coli* expression fails. A protein important for pharmaceutical research stimulated our effort to embark on finding methods for preparation of isotopically labeled proteins in the Baculovirus system, and thereby extend the scope of proteins amenable to NMR studies. In this communication, we disclose an efficient and inexpensive way to produce proteins with the Baculovirus expression system, in which a single amino acid type is isotopically labeled, and as well we demonstrate the usefulness of these selectively labeled proteins for NMR studies.

c-Abl kinase is a tyrosine kinase and the target protein of the anti-leukemia drug STI-571 (Glivec®) (Fabbro et al., 2002). A crystal structure of the catalytic domain of c-Abl kinase with a derivative of STI-571 has been published (Schindler et al., 2000). Special interest in the structure and dynamics of c-Abl kinase exists for a better understanding of Glivec's mode of action and the design of a further improved drug. Unfortunately, the catalytic domain of c-Abl kinase cannot be expressed in *E. coli* well enough for NMR studies. As is typical for kinases, it can be successfully expressed only in eucaryotic cells such as Baculovirus-infected insect cells. Since in insect cells c-Abl kinase exhibits a good expression behaviour (sufficient expression levels of soluble and qualitatively good protein), we have chosen to attempt the isotope labeling of the catalytic domain of c-Abl kinase in Baculovirus-infected insect cells.

A construct of the catalytic domain of human c-Abl kinase with Baculovirus was used, leading to expression in insect cells of predominantly one protein of 299 amino acids and a molecular weight of 34893.8 Da. This construct begins at the N-terminus with (M)S $*$ YYHHHHHHDYDIPTTENLYFQGAMDPSP NYD... (starting methionine cleaved off; S$*$ appears as N-acetyl-Serine; includes 6His-tag/linker/TEV-protease site/GAMDP/S^{229}-S^{500}), referred herein subsequently as 6His-GAMDP-Abl(229-500). At purification the 6His-tag was cleaved off, leading mainly to the unphosphorylated c-Abl protein used for NMR analysis with 277 amino acids and a molecular weight of 32014.7, starting N-terminally with GAMDPSPNYD... (GAMDP/S^{229}-S^{500}), referred herein subsequently as GAMDP-Abl(229-500).

Recombinant Baculovirus was produced by cloning the gene region coding for h c-Abl(229-500) into a pFastBac HTa donor plasmid (Bac-to-Bac system of Invitrogen) at the BamH I restriction site, and subsequent transformation of competent DH10Bac *E. coli* cells with it, transposition, selection and amplification of the rec.(recombinant) Bacmid in *E. coli* (Lac7-), and transfection of Sf9 cells with the rec. Bacmid DNA according to the manufacturers instruction. Following the protocols for Baculovirus expression methods given by O'Reilly et al. (1994), the rec. Baculovirus was plaque-cloned and amplified in a second amplification round in attached Sf9 cells cultured at 27 °C in 25 cm^2-TC-flasks with 10 ml medium TC100 (Invitrogen) + 10%FCS (Fetal Calf Serum; Invitrogen). A third amplification round was done in suspended Sf9 cells cultured at 27 °C, shaken at 90 rpm in 500 ml-Erlenmeyer flasks with 100 ml medium TC100 + 10% FCS + 0.1% Pluronic F-68 (Invitrogen). The resulting rec. Baculovirus suspension had a titer of 1.91×10^8 pfu ml^{-1}, determined by plaque assay.

Successful aasi-labeling of biologically expressed proteins is mainly dependent on (a) sufficient expression of soluble and good quality rec. protein under labeling culture conditions, (b) a minimal label dilution, resulting in a sufficiently high incorporation rate (>90%) of the labeled amino acid into the protein, (c) a negligible cross-labeling of other amino acids. To avoid excessive label dilution, cultured cells have to be, at the moment of expressing the rec. protein in a culture medium, devoid of an unlabeled form of the respective amino acid. However, most of the insect cell culture media commonly used for rec. protein expression contain large amounts of growth-promoting but undefined sources of amino acids, such as yeast extract (YE) or protein hydrolysate. We therefore tested several culture media suitable for labeling (without undefined amino acid sources) and insect cell culture regimes for growth and expression of c-Abl kinase in 50 ml-shake flask cultures. Starting from the commercially available insect cell culture medium SF-4 Baculo Express ICM (Bioconcept, Amimed, #9-00F38, www.bioconcept.ch), here called in short SF-4, various versions of SF-4, devoid of an undefined amino acid source were produced by Bioconcept, e.g., SF-4/C (SF-4 without YE), SF-4/C-w/oPhe (SF-4 without YE, with all amino acids except L-phenylalanine) and SF-4/B (SF-4 without YE and without amino acids). SF-4-derived media depleted of YE and none, one, several or all amino acids are commercially available on request from Bioconcept. Labeled media, such as SF-4/C-^{15}N-Phe (SF-4 without YE, with ^{15}N-phenylalanine and all other amino acids unlabeled) were prepared by adding the labeled amino acid (e.g.,

^{15}N-phenylalanine) to the medium lacking this amino acid (e.g., SF-4/C-w/oPhe).

For testing media and culture conditions, suspended Sf9 cells, adapted to growth in serum-free medium were precultured at 27 °C and 90 rpm for three days in 100 ml medium SF900 II (Invitrogen) in 500 ml-Erlenmeyer flasks to reach a cell density of ca. 3×10^6 cells ml^{-1}. Main cultures with a final volume of 50 ml culture with SF-4 medium in 200 ml-Erlenmeyer flasks were inoculated in general directly with 5 ml of the preculture and grown for ca. 3 days at 27 °C, shaken at 90 rpm. In case of direct growth and expression in SF-4/C medium, pelleted cells from 5 ml preculture taken up in 50 ml of SF-4/C were used for inoculation. When a cell density of 1.5×10^6 cells ml^{-1} was reached, cultures were centrifuged at 300 *g* at rt (room temperature) for 10 min, cell pellets were taken up in 50 ml fresh medium for expression and 0.4 ml of rec. Baculovirus suspension of a titer of 1.91×10^8 pfu ml^{-1} giving a MOI = 2 (MOI = multiplicity of infection) was added. The cultures were then further incubated for 3 days p.i. (post infection) under the same conditions. In case of direct expression in SF-4/C medium, rec. Baculovirus infection was performed without medium change. At harvest, 1 ml-samples for SDS-PAGE analysis and 30 ml-samples for cell fresh weight (fw) determination were taken and centrifuged at 300 *g* at rt for 10 min. Cell pellets from the 1 ml-samples were taken up in 1 ml 1 × SDS-PAGE buffer and an amount of such total lysates normalized for cell yield (0.2 mg fw cells) was applied on the gels for SDS-PAGE analysis. Results from these experiments, presented in part in Figure 1, show that when Sf9 cells are first grown in a 'full' medium, such as SF-4 and then prior to viral infection transferred by centrifugation to a medium potentially suitable for labeling, such as SF-4/C, c-Abl-expression and cellular yield are almost as high (lanes 3 and 6) as when cells are resuspended again in SF-4 (lane 7) and are thus sufficient (ca. 10 mg l^{-1} 6His-GAMDP-Abl(229-500) expressed calculated by gel densitometric evaluation in comparision to reference material, lane 9) for successful labeling. With regard to protein quality, 6His-GAMDP-Abl(229-500) expression is manifested by a clearly visible band on a Coomassie-stained gel of correct molecular weight and a predominant signal on a Western blot, treated with anti-cAbl-antibody at the corresponding size, with only few cross-reacting material (Figure 1). The minor cross-reacting bands on Western blots have not been analysed further; they presumably repre-

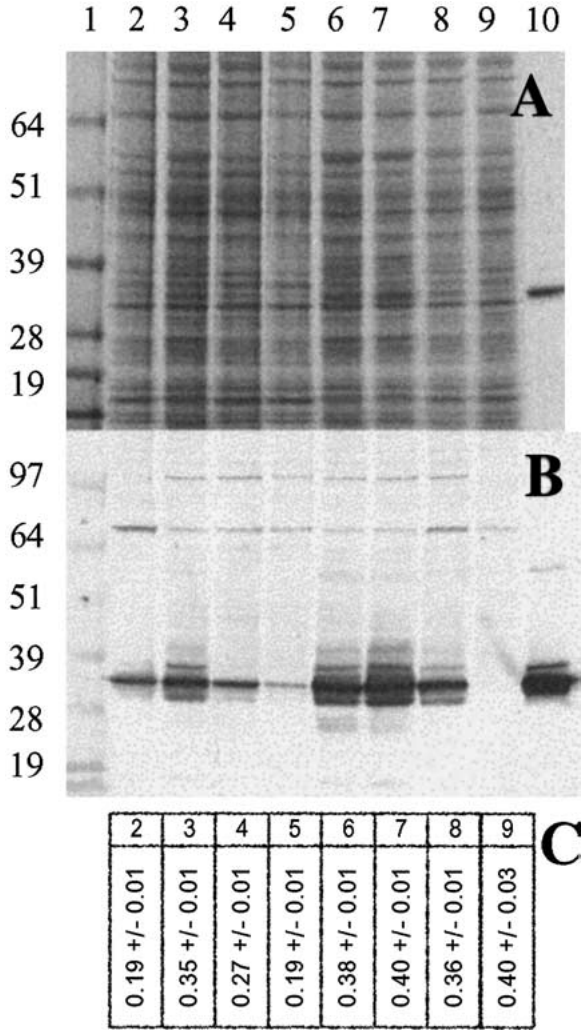

Figure 1. c-Abl-expression analysed by SDS-PAGE (A,B) and cellular yield (C) from 45 mL-shake flask cultures with Sf9 cells in various media, harvested 3 days p.i. as described in the text above. (A,B) SDS-PAGE analysis of samples with total lysates from 0.2 mg fresh weight cells (lanes 2-9) or 0.84 μg purified reference 6His-GAMDP-Abl(229-500) (lane10); Molecular weight marker (Invitrogen, SeeBlue Plus2) with given size in kDa (lane 1); Sf9 cells cultured in SF-4/C without medium change (lane 2) or cultured in SF-4 with medium change to SF-4/C (lane 3), to SF-4/C-w/oPhe (lane 4), to SF-4/B (lane 5), to SF-4/C-^{15}N-Phe (lane 6), to SF-4 (lane 7), to IPL-41 (lane 8) and to SF-4/C but infected with mock virus (lane 9). (A): Coomassie-stained gel; (B): Western blot treated with anti-cAbl-ab (Oncogene, # OP19-100UG); (C): Cellular yield given as mean fresh weight of cells in g from 30 mL culture (n = 3) with standard deviation. Sample numbering as for (A,B)

sent proteolytic degradation products (smaller size) or post-translationally modified versions (bigger size) of the expressed c-Abl kinase. 6His-GAMDP-Abl(229-500) is expressed largely in a soluble form (data not shown). In contrast, continuous culturing of cells in SF-4/C without medium change results in poor c-Abl-expression and cellular yield and is thus unsufficient for labeling (lane 2). Supplementing second medium SF-4/C with vitamines, additional amino acids or dialysed FCS does not further improve c-Abl-expression (data not shown). Omission of all amino acids or one single amino acid (here shown for L-phenylalanine) leads to reduced cellular yields and residual c-Abl-expression (lanes 4 and 5), indicating that the cell internal pool of unlabeled amino acids is rather small under the conditions used. Appreciable levels of c-Abl are also expressed when Sf 21 cells were used (data not shown) or when cells were transferred to other media with a defined amino acid source, such as IPL-41 (Weiss and Vaughn, 1986) (lane 8).

Growth of insect cells in first medium SF-4 and medium change to second medium SF-4/C (or the corresponding versions with only one amino acid labeled) was therefore chosen as culture conditions for production at the liter scale of sufficient aasi-labeled c-Abl kinase for NMR analysis. Sf9 or Sf21 cells adapted to SF900 II medium were precultured at 27 °C and 90 rpm for three days in 100 ml medium SF900 II in 500 ml-Erlenmeyer flasks, and 50 ml of this was used to inoculate the main culture with 800 ml SF-4 medium in a 5 l-Erlenmeyer flask. After ca. 3 days culturing of the main culture at 28 °C and 90 rpm, when cells have reached a density of 1.5×10^6 cells ml^{-1}, cultures were transferred to 1L-centrifuge bottles and were centrifuged at 400 *g* at rt for 10 min. The supernatant was discarded as completely as possible, cell pellets were resuspended in second medium for labeling, e.g., SF-4/C-^{15}N-Phe, transferred back to the 5 L-Erlenmeyer flasks and 13 ml of rec. Baculovirus suspension of a titer of 1.91×10^8 pfu ml^{-1} giving a MOI = 2 was added, and the cultures were further incubated for 3 days p.i. under the same conditions. In the cases mentioned later, for suppression of phosphorylation of c-Abl, 0.8 ml of 10 mM STI-571 dissolved in DMSO was diluted in 200 ml of the labeling medium, filter-sterilized and added to the culture, giving a final concentration of 10 μM. c-Abl-expressing cells were harvested by two subsequent centrifugations at 400 *g* at rt for 20 min first in 1 L-centrifuge bottles and then after having taken up the pelleted cells in 20 ml PBS, pH 6.2 with a protease inhibitor mix (Complete^{TM}, Roche) in 50 ml-plastic tubes and frozen and stored at −80 °C prior to extraction.

Cells were lysed by mild sonication in 50 mM Na-phosphate, 300 mM NaCl, 10% by vol. glycerol, 10 mM 2-mercaptoethanol, pH 8, supplemented with a protease inhibitor cocktail. For cells from cultures done without STI-571, the lysis buffer contained in addition 25 μM STI-571. After clarification by centrifugation and filtration the lysate was applied to a column of Ni-NTA superflow resin (Quiagen). The bound 6His-Abl/STI-571 complex was eluted with 100 mM imidazole in Tris-buffer, 100 mM NaCl, pH 8 and incubated with TEV-protease (Invitrogen) at 4 °C overnight to remove the His-tag. The cleavage reaction was purified on a Mono Q anionexchange column (Amersham Biosciences) using a gradient from 50 to 300 mM NaCl in 20 mM Tris, 5% by vol. glycerol buffer, pH 8. The peak containing unphosphorylated c-Abl/STI-571 was concentrated and applied to a Superdex 75 size exclusion column (HiLoad 16/60, Amersham Biosciences) run in 20 mM Bis-Tris,100 mM NaCl, 3 mM DTT, pH 6.5 (21 °C). The c-Abl/STI-571 complex eluted at the position corresponding to the monomeric protein. Purification steps were analysed by reversed phase HPLC to determine the concentrations of c-Abl and STI-571, the samples used for the NMR studies showed a molar ratio of about 1:1. The final yield of homogeneous unphosphorylated GAMDP-Abl (229–500) obtained from the four aasi-labeling culture runs described were 3 to 5 mg l^{-1}. The purified c-Abl samples were subjected to LC-MS analysis to determine the amount of ^{15}N-label incorporated.

For NMR analysis, c-Abl(229-500) was concentrated to about 14 mg ml^{-1} (0.4 mM) in a buffer containing 20 mM Bis-Tris, pH 6.5 (21 °C), 100 mM NaCl, 0.5 mM EDTA, and 3 mM DTT. Alternative conditions were 20 mM Tris, pH 8 (4 °C), 100 mM NaCl, 0.2 mM EDTA, 3 mM DTT. NMR spectra were acquired at 23 °C with a 600 MHz Varian UnityPlus or 800 MHz Bruker DRX spectrometer.

Aasi-labeling of c-Abl kinase was performed in the described way for L-phenylalanine (^{15}N-Phe, 1000 mg/l; CIL, #NLM-108, ^{15}N, 98%+) and for glycine (^{15}N-Gly, 200 mg/l; CIL, #NLM-202,^{15}N, 98%+), both with Sf9 cells in the presence of STI-571 and for L-tyrosine (^{15}N-Tyr, 360 mg l^{-1}; CIL, #NLM-590,^{15}N, 95-99%) and for L-valine (^{15}N-Val, 500 mg l^{-1}; CIL, #NLM-316, ^{15}N, 95–99%), the latter two both with Sf21 cells in absence of STI-571 (Table 1).

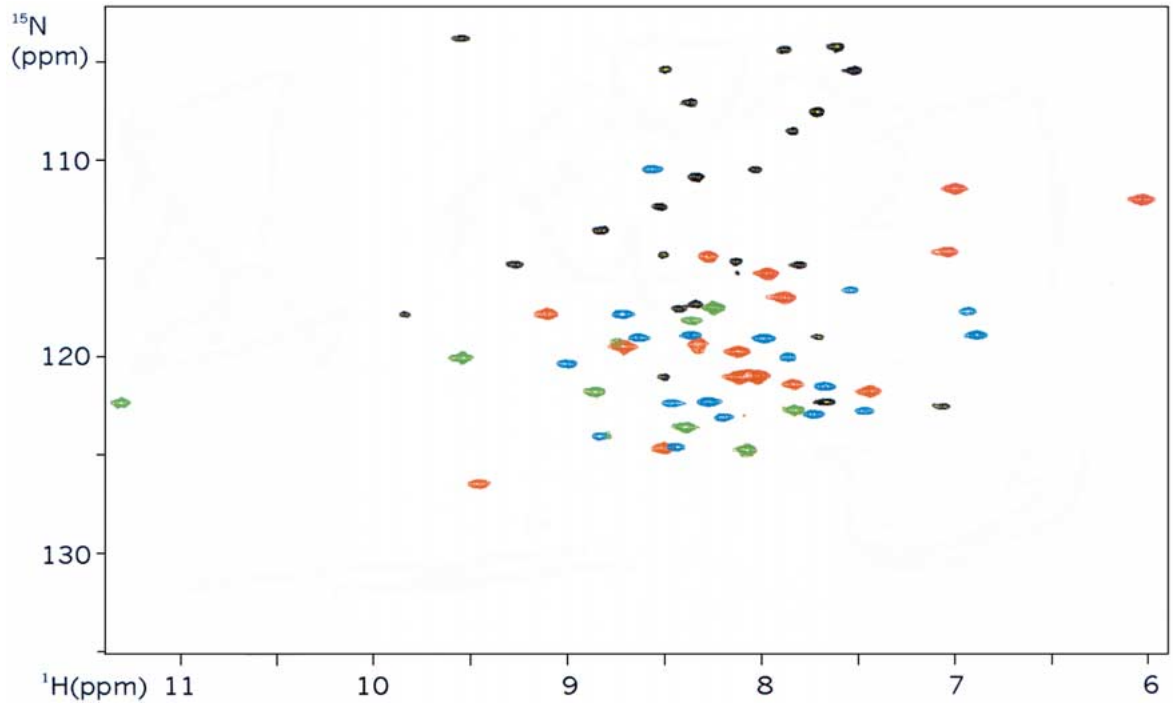

Figure 2. [^{15}N;^{1}H]–HSQC spectra for cAbl kinase, labeled with ^{15}N-Phe (in green), ^{15}N-Tyr (in red), ^{15}N-Val (in blue) or with ^{15}N-Gly (in black).

Table 1. Yields at purification, label incorporation and number of resonances in HSQC spectra for c-Abl kinase labeled with ^{15}N-Phe, ^{15}N-Tyr, ^{15}N-Val or ^{15}N-Gly

Amino acid labeled	^{15}N-Phe	^{15}N-Tyr	^{15}N-Val	^{15}N-Gly
Host cell[a]	Sf9	Sf21	Sf21	Sf9
c-Abl (mg l^{-1})[b]	9	5	12	10
Number of aa residues[c]	11	16	18	16
Number of resonances[d]	10	16	18	23
Incorporation rate %[e]	92	90	95	78

[a]c-Abl-expressing Sf9 cells cultures in presence of 10 μM STI-571.
[b]6His-GAMDP-Abl(229–500) isolated by Ni-chelate chromatography.
[c]Number of amino acid residues in GAMDP-Abl(229–500).
[d]Observed number of signals in [^{15}N;^{1}H]-HSQC spectra.
[e]Incorporation rates for the ^{15}N-labeled amino acids into GAMDP-Abl(229–500) are calculated as percentage of the observed to the theoretical mass increase in MS spectra.

MS-spectra of purified GAMDP-Abl(229–500) for all four tested cases of aasi-labeling (^{15}N-Phe; ^{15}N-Gly; ^{15}N-Tyr and ^{15}N-Val) show a prevailing mass peak in the expected range of molecular weight. For the essential amino acids Phe, Tyr and Val, the incorporation rates were all found to be at least 90%, as shown in Table 1, demonstrating efficient incorporation of the ^{15}N-labeled amino acids into c-Abl kinase. For the non-essential amino acid Gly, the apparent incorporation rate of 78% is distinctly lower. Considering the data from the HSQC-spectrum (Figure 2), suggesting cross-labeling of other amino acids, the true incorporation rate of ^{15}N-Gly might be even lower. The main reason for the lowered incorporation rate for ^{15}N-Gly might be label dilution by conversion of a non-labeled, metabolically related amino acid (e.g., Ser) into Gly.

The HSQC-spectra of c-Abl kinase aasi-labeled for ^{15}N-Phe, ^{15}N-Gly, ^{15}N-Tyr, or ^{15}N-Val exhibit a pattern of well-dispersed, sharp resonances (Figure 2). Furthermore, all the observed resonances match well with resonances from a HSQC spectrum from uniformly ^{15}N-labeled c-Abl kinase (unpublished results). The number of resonances observed for the essential amino acids Phe, Tyr and Val correspond well to the expected number, indicating that only the intended amino acids were substantially labeled. Labeling of c-Abl kinase with the non-essential amino acid ^{15}N-Gly gives 23 instead of the expected 16 res-

onances in the HSQC-spectrum (Figure 2); additional signals might stem from cross-labeling of other amino acids, metabolically related to Gly, such as Ser.

If the amino acid type to be labeled is properly designed, an aasi-labeled protein can essentially be used for the same NMR purposes as uniformly labeled proteins, e.g. for the validation of ligand binding, rough determination of the binding site, K_D determination by ligand titration, or detection of protein conformational changes upon ligand binding (Pellecchia et al., 2002). With material costs often as low as a few hundred US dollars for 10 mg of protein, one then has most of the NMR options as with a fully labeled (*E. coli*) protein. An aasi-labeled protein can even be advantageous since there is essentially no resonance overlap, which can severely hinder the analysis in fully labeled proteins. Moreover, resonance assignment is significantly aided by knowledge of the respective amino acid type (Weigelt et al., 2002). The presented method for aasi-labeling of proteins with ^{15}N-labeled amino acids has also been successfully applied to another rec. protein expressed with Baculovirus-infected insect cells (unpublished results). A wider application for isotope labeling of other proteins expressed with Baculovirus is thus possible, if expression levels and protein quality allow. Furthermore, the present study shows, that this labeling method functions irrespective of the used host strain, as aasi-labeling was successfully applied for the two different insect cell lines Sf9 and Sf21.The observed high label incorporation rates at least for essential amino acids opens the possiblity for successful aasi-labeling of rec. proteins with ^{13}C/^{15}N-labeled amino acids with the Baculovirus expression system, useful for resonance assignment and further NMR analysis. NMR analysis of solution structure and dynamics of the catalytic domain of c-Ab1 kinase is currently underway in our laboratory.

Acknowledgements

We are grateful to Silvie Antz, Carmen Huesa and Paul Rheinberger for their experimental contributions and to Drs Sabine Geisse and Marcel Blommers and the colleagues of the Protein Structure Unit of CT, Novartis Pharma AG, for the fruitful discussions and comments.

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