Journal of Biomolecular Structure & Dynamics, ISSN 0739-1102
Volume 26, Issue Number 6, (2009)

A Cost-effective Amino-acid-type Selective Isotope Labeling of Proteins Expressed in *Leishmania tarentolae*

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Abstract

We report a cost efficient approach for amino-acid-type selective isotope labeling of proteins expressed in *Leishmania tarentolae*. The method provides an economically advantageous alternative to recently established protocol for isotopic labeling using expensive synthetic media. The method is based on cultivation of the *L. tarentolae* expression strain in a cheap complex medium supplemented with labeled amino acid(s). In this protocol, a labeled amino acid is deliberately diluted in the medium of undefined composition, which leads to a low-level isotope enrichment upon protein over-expression. The economic advantage of the protocol is achieved by avoiding large volumes of expensive synthetic medium. Decreased sensitivity of a NMR experiment due to low-level isotope enrichment is compensated by a five- to seven-fold increase of the yield of the recombinant protein in complex medium as compared to that in the synthetic medium. In addition, the decreased sensitivity can be compensated by using a higher magnetic field, cryo-detection system or higher number of transients during the NMR data acquisition. We show that enrichment as low as 5% does not compromise a NMR experiment and makes preparation of the recombinant proteins over-expressed in *L. tarentolae* economically viable. The method is demonstrated by selective labeling of the ~27 kDa enhanced green fluorescent protein (EGFP) with ^{15}N-labeled valine.

Key words: NMR; Isotope labeling; Protein expression; *Leishmania*; Low-level enrichment.

Introduction

A majority of biomolecular NMR techniques requires isotopic (^{2}H, ^{13}C, ^{15}N) labeling of recombinant proteins. Currently, most isotopically labeled recombinant proteins are expressed in the bacterial host *Escherichia coli*. Despite the obvious advantages of this system such as rapid growth, well-developed methods of proteins expression, cheap cultivation, and isotopic labeling, *E. coli* has a range of shortcomings that limit its utility in protein studies. The most prominent problem relates to the inefficiency of *E. coli* to assist folding of eukaryotic proteins, producing only about 15% of them in their native form (1). Moreover, prokaryotic expression systems lack the post-translational modifications essential for functionality of many eukaryotic proteins. If over-expression in *E. coli* is not feasible due to protein insolubility or bacterial toxicity, other expression systems, such as *Pichia pastoris* (2-7), Chinese hamster ovary (CHO) cells (8, 9), or baculovirus-infected insect cells (10-13) can be used for producing isotopically labeled proteins [reviewed in (14, 15)]. Alternatively, cell-free expression or chemical synthesis is sometimes also feasible (16-21). However, if mammalian-like post-translational modifications also have to be taken into account, the choice of the expression system is essentially reduced to the CHO cells. Production of isotopically labeled proteins in these cells makes use of a serum-free CHO medium, which initially lacks amino acids and carbohydrates. Isotopic labeling is achieved by supple-

menting the medium with either ^{15}N and/or ^{13}C-labeled amino acid(s) for uniform incorporation into *de novo* expressed proteins (8, 9). For some proteins, however, the CHO mammalian expression system does not produce the appropriate yields to make isotopical labeling economically viable. Thus, alternative routes for the production of isotopically labeled proteins that also possess mammalian-like post-translational modifications are of general interest.

Recently, a novel protein expression system based on the non-pathogenic trypanosomatid flagellate *Leishmania tarentolae* was introduced (22, 23). In addition to easy manipulation, rapid growth, and relatively high-yield protein production, this expression system proved to be an alternative to mammalian cell as it provides complete eukaryotic folding machinery and mammalian-like protein glycosylations. For NMR studies, isotopically labeled proteins can be produced by growing the cells in a synthetic medium with defined composition that is supplemented with labeled amino acids (24). However, in analogy to the CHO cells, there are two practical limitations of this approach. First, as a consequence of the transfer into a synthetic medium, the growth rate of *L. tarentolae* significantly slows with a parallel decrease of over-expression of the recombinant protein. Second, due to the low yield of recombinant protein, large culture volumes are required. Expenses associated with the use of synthetic media and isotopic labels thus become substantial.

In general, the applicability of the *L. tarentolae* expression system for production of recombinant proteins for NMR structural studies requiring uniform isotopic labeling is rather limited due to the high sample costs. However, the *L. tarentolae* expression system can be used, with its full advantage of complete eukaryotic folding machinery and mammalian-like protein glycosylations, for many NMR studies such as investigation of ligand binding, evaluation of influence of post-translational modification on protein folding, or verification of proper folding in crystallization studies. In these cases, uniform labeling is not required and the costs can be notably reduced by employing an amino-acid type selective (AATS) isotope-labeling scheme (25). Within the AATS scheme, the medium is supplemented with a single or very limited set of isotopically labeled amino acids. Despite the fact that the use of the AATS labeling reduces sample costs by decreasing the amount of isotopic labels used, the AATS sample from *L. tarentolae* preparation remains quite expensive, mainly because of the relatively low yields of recombinant proteins in the synthetic medium. The production of the isotopically labeled NMR sample requires the use of large volumes, typically > 10 liters, of expensive synthetic medium.

To allow for cost-effective preparative production of AATS labeled proteins for NMR studies using the *L. tarentolae* expression system, we adopted the concept of low-level isotopic enrichment by Phan and Patel originally developed for low-level isotope enrichment of nucleic acids (26). The method is based on cultivation of *L. tarentolae* in a cheap, complex medium supplemented with labeled amino acid(s). In this protocol, a labeled amino acid is deliberately diluted in a medium of undefined composition, which leads to a low-level AATS isotope enrichment of the over-expressed protein. We show that the notable economic advantage of this protocol can be obtained by considering differences between the protein yields in synthetic and complex media in conjunction with properly estimated minimal concentration of isotopic label required for achieving reasonable sensitivity in a NMR experiment.

Materials and Methods

Construction of the Expression Strain

The *L. tarentolae* T7-TR strain, constitutively expressing both T7 RNA polymerase and TET repressor from the chromosomal rDNA locus was used for expression experiments (Kushnir *et al.*, 2005). This strain was transfected with an episomal EGFP expression vector, pLAC-EGFP#01226 (Cirstea & Alexandrov,

unpubl. results), and the recombinant clones were selected on BHI agar plates (Jena Bioscience, LEXSY protocols).

^{15}N-valine Labeled EGFP Expression and Purification

For labeled protein production, the recombinant strain was initially grown in LEXSY BHI (Jena Bioscience) at 26 ºC with shaking at 80 rpm. After the optical cell density at 600 nm (OD_{600}) reached 1.5, the cells were pelleted by 10 min centrifugation at 2500 g. The pellet was then resuspended to an equivalent OD_{600} of about 0.7 in fresh BHI medium supplemented with 150 mg/L ^{15}N (98%)-labeled valine. Protein production was induced by the addition of 5 mg/L tetracycline and the culture was incubated at 26 ºC with shaking at 80 rpm for an additional 24 hrs. The cells were then pelleted by centrifugation for 10 min at 2500 g and resuspended in 20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, and 1 mM PMSF. Next, the slurry was subjected to ultrasonic homogenization followed by flash freezing in liquid nitrogen, which was repeated three times in total. The homogenate was cleared by centrifugation for 1 hr at 30,000 rpm. The EGFP was purified from the supernatant by organic extraction, as described elsewhere (27).

Effect of α-amanitin on Cell Growth and Protein Expression

To evaluate the effect of suppression of endogenous protein synthesis by α-amanitin, cells were grown in the BHI medium in the presence or absence of the drug, while leaving all other parameters unchanged. Different concentrations of α-amanitin (3 mg/L, 7 mg/L, 10 mg/L, 15 mg/L) were added to the media of individual cultures in parallel with tetracycline (5 mg/L). The growth of untreated and drug treated cultures were measured over a period of 7 days.

For Western blotting, protein lysates were analyzed on 12% SDS-PAGE, blotted onto nitrocellulose membrane and probed with anti-EGFP antibodies as described elsewhere (28).

NMR Spectroscopy

The heteronuclear sensitivity enhanced ^{1}H-^{15}N TROSY spectra (29, 30) were collected on a Bruker Avance 700 MHz spectrometer equipped with z-gradient shielded triple-resonance cryogenic probe head. The spectra were acquired at 298 K with 64 scans and 2048 × 256 complex data point in t_2 and t_1, respectively. The total measurement time was 5 hrs. The spectra were processed using the standard Bruker NMRSuite programs.

Mass Spectrometry

To evaluate the level of isotope enrichment, the unlabeled and AATS-labeled EGFPs were subjected to digestion by trypsin for a period of 48 hours. The protein digests were desalted using ZipTip$_{C18}$® (Millipore, USA) and undigested EGFP was removed by ultra-filtration. The mass of EGFP peptides was measured using a Waters *nano*UPLC-qTOF *Premiere* mass spectrometer. The data were processed and analyzed with the PLGS1.0 program (Waters, UK). The level of isotope enrichment was estimated from mass differences between corresponding labeled and unlabeled EGFP peptides.

Results and Discussion

The T7-TR strain of *L. tarentolae* transfected with the pLAC2-EGFP #01226 vector was used for production of recombinant EGFP. The *L. tarentolae* cells were grown in BHI medium in the absence of tetracycline and then transferred into a fresh BHI medium supplemented with 150 mg/L ^{15}N-labeled valine (for details

see ***Materials and Methods***). One hour after the transfer, protein production was induced by the addition of tetracycline and the cells were cultivated for an additional 24 hours. Next, the cells were subjected to ultrasonic homogenization and the labeled EGFP was purified from cleared homogenate by organic extraction (for details see ***Materials and Methods***).

Figure 1A shows a comparison of growth rates of *L. tarentolae* in the complex BHI and synthetic media over the period of 24 hours, corresponding to typical time-course of over-expression of recombinant proteins in this flagellate. After 16 and 24 hours of cultivation, the cells grown in BHI medium were 2.5- and 5-fold more dense, respectively, than those in the synthetic medium at the same time points. Moreover, the normalized protein expression rate achieved in the BHI medium was also higher, resulting in about 30-35% higher yield of the expressed protein after 24 hours of cultivation (Figure 1B).

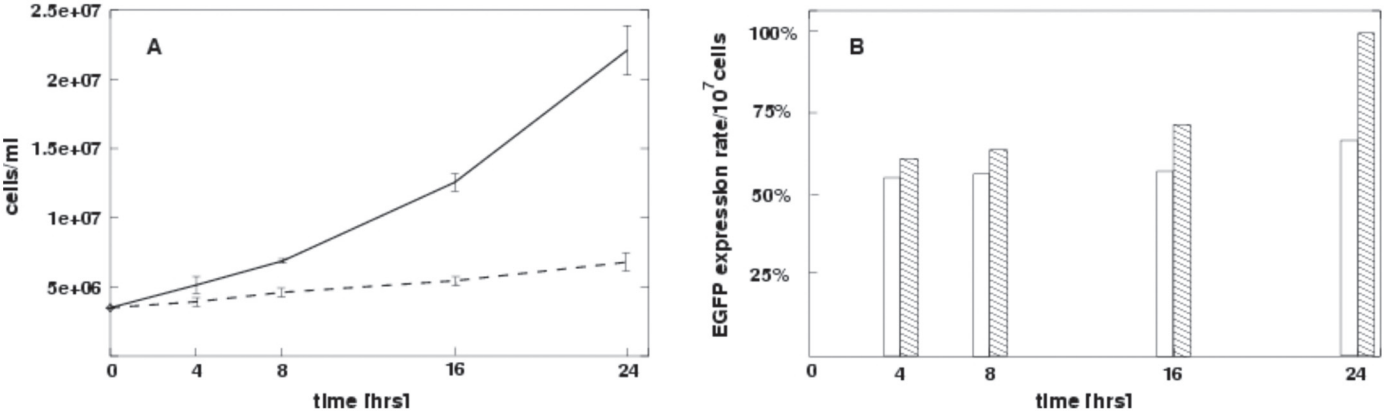

Figure 1: (**A**) Relative *L. tarentolae* growth rates in the BHI medium (solid line) and Synthetic LEXSY (dashed line) media. (**B**) Relative EGFP expression rate in Synthetic LEXSY Broth (open box) and the BHI (stripped box) media. The expression rates were normalized to 10^7 cells per ml.

The isotopic labeling of the EGFP was performed by supplementation of the BHI medium with 150 mg/L ^{15}N-labeled valine. This approach inevitably led to an excessive dilution of the label, and consequently to its rather low incorporation into the over-expressed protein, since the complex BHI medium contains undefined sources of amino acids. Mass spectrometric (MS) analysis revealed that growth of *L. tarentolae* in the BHI medium supplemented with 150 mg/L of ^{15}N-labeled valine resulted in ~4.5% enrichment of recombinant EGFP by ^{15}N-labeled valine.

To increase the level of selective incorporation of the isotopic label into the protein to be over-expressed, we attempted to decrease the expression of endogenous proteins by selective suppression of RNA polymerases II and III by α-amanitine. In contrast to endogenous proteins, those to be over-expressed are under control of the T7-polymerase, which is not affected by the drug. RNA polymerases II and III were shown to be inhibited by α-amanitine at concentrations higher than 2 μg/L (31-33). To evaluate the effect of suppressing the endogenous protein production by α-amanitine, we have expressed EGFP in the presence and absence of the drug while leaving all other parameters unchanged. The lysates from both preparations were subjected to SDS PAGE analysis. Western blots revealed that a concentration of α-amanitine as high as 15 μg/L did not significantly inhibit endogenous proteins synthesis (data not shown). Treatment with α-amanitine was also found to have only a moderate effect on the growth rate of *L. tarentolae*. A plausible explanation for these observations might be an inadequate dosage of α-amanitine used for treatment of *L. tarentolae* cells. The concentrations of α-amanitine required for inhibition of polymerases II and III were determined using nuclear run-on assay (31, 34). For intact cells, much higher concentrations of the α-amanitine would be most probably required to reach the same inhibitory effect. However, the use of high concentration of α-amanitine would also significantly increase the cost of sample preparation.

Figure 2 shows the two dimensional (2D) ^{1}H-^{15}N HSQC spectrum of the purified

EGFP enriched to ~4.5% by ^{15}N-valine. Eighteen major resonances were expected to be found in the ^{1}H-^{15}N HSQC spectra, as the EGF protein variant used in this study contains 18 valine residues. Indeed, based on previously published assignments (24, 35), we were able to assign all 18 of the EGFP valine resonances. [Although, the signal corresponding to valine No. 5 (circled) was very weak.] In addition to the 17 strong EGFP resonances, several strong peaks were observed in the frequency region of 7.9-8.1 ppm (^{1}H) and 118-124 ppm (^{15}N) (boxed area in Figure 2). These resonances are likely to arise from degradation/denaturation of the protein during measurement, as the chemical shifts of these resonances are close to the random coil shift found for valine residues (36). Alternatively, they may represent a minor fraction of a co-purified unfolded protein. In general, the overall appearance (including boxed area) and quality of the ^{1}H-^{15}N HSQC spectrum is very similar to the spectrum obtained for a fully labeled sample (24).

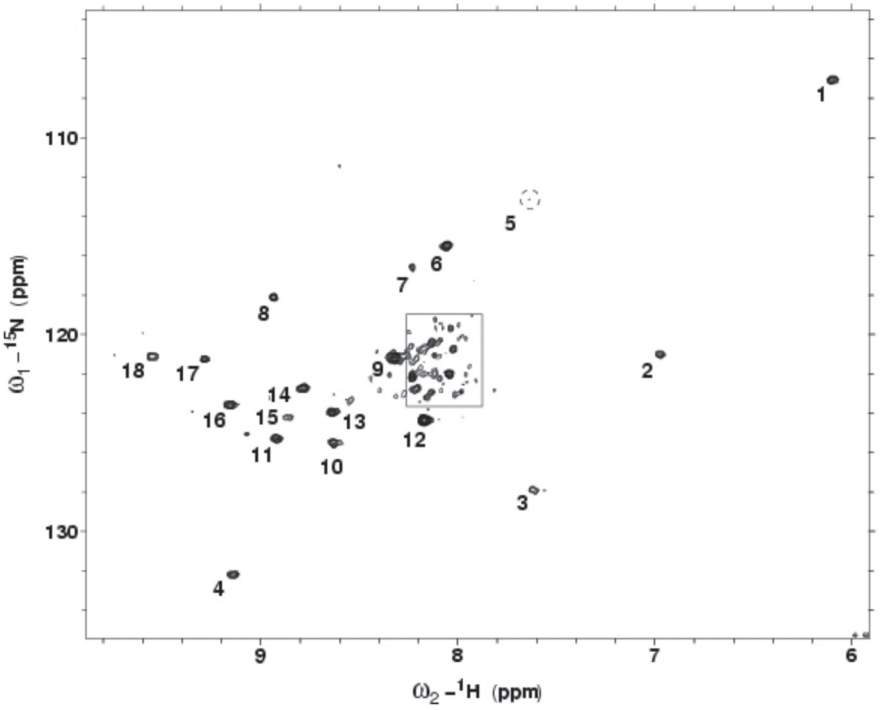

Figure 2: Heteronuclear sensitivity enhanced 2D ^{1}H-^{15}N TROSY spectrum of 0.9 mM ^{15}N-Val labeled EGF protein in 90% $^{1}H_2O$/10% $^{2}H_2O$, 50 mM NaPi, pH=7 at 298 K acquired on a 700 MHz Bruker Avance NMR spectrometer. Resonances marked by the frame exhibit chemical shifts close to the random coil shift of valine amide groups observed in small unstructured peptides, and thus likely to arise from protein degradation or an unfolded protein.

Taken together, our data suggest that the use of the complex BHI medium is superior to the synthetic medium, as far as protein yield is concerned. Further, the data shows that isotope enrichment as low as 4.5% does not compromise the quality of NMR spectra. Most importantly, application of this method significantly reduces the overall costs. In our case, the NMR sample (~12.2mg of EGFP enriched to 4.5% by ^{15}N-valine, dissolved in 500 µl; ~0.9 mM) was prepared out of 6 liters of the BHI medium and a total 900 mg of ^{15}N-valine with overall costs of 220 EUR. It should be mentioned that the sample costs could be further reduced by decreasing volume of the NMR sample. In contrast, a previous preparation of the labeled NMR sample (~5 mg of EGFP enriched to 98% with ^{15}N-valine in 250 µl; ~0.65 mM) required 10 liters of synthetic LEXSY medium supplemented with 250 mg of ^{15}N-valine (24). At current prices, this corresponds to overall costs about 1550 EUR. The NMR time (~5 hrs) required for ^{1}H-^{15}N HSQC spectrum acquisition of 4.5% enriched EGFP NMR sample was essentially the same as the time required for recording of the same spectrum on the 98% labeled sample by Nicuale *et al*. (2006). However, in this case, the low sensitivity of NMR experiment resulting from low-level isotope enrichment was compensated by about a 30% higher concentration of low-level enriched NMR sample and the use of 700 MHz NMR spectrometer with cryo-detection system as compared to 500 MHz NMR spectrometer used in original study by Niculae *et al*. (2006).

This example shows that the loss of sensitivity due to low-level enrichment of the

Table I
The amount of the essential amino acid to be added to the BHI medium for achieving of about 5% amino acid type selective isotope labeling. The estimate is based on experimentally determined value for valine and relative abundances of amino acids in proteins. Essential amino acids are printed in bold.

Amino acid	% abundancea	Est. amount of EAA in BHI medium [mg/L]
Trp	1.34	30
Cys	1.76	-
His	2.26	60
Arg	5.20	120
Met	2.32	60
Tyr	3.25	75
Gln	3.96	-
Phe	4.12	90
Asp	5.12	-
Pro	5.00	-
Ala	7.34	-
Asn	4.57	-
Lys	5.81	135
Glu	6.22	-
Gly	6.89	-
Thr	5.85	135
Val	6.48	150
Ile	5.76	-
Ser	7.38	165
Leu	9.36	210

[a]Betts, M. J., Russell, R. B. Amino acid properties and consequences of subsitutions. In Bioinformatics for Geneticists, M. R. Barnes, I. C. Gray eds, Wiley, 2003

over-expressed protein can be outweighed by the increased yield of the protein and, if required (*e.g.*, there is a problem with a protein solubility), can be further compensated by using a higher magnetic field, cryo-detection system, or higher number of transients during the NMR data acquisition.

In principle, the level of both label dilution and the enrichment can be modulated by the amount of labeled amino acid(s) added to the BHI medium. However, from practical point of view, one wants to keep the amount of label at minimum to reduce the sample costs and to keep osmotic strength at reasonable ranges as it might influence a growth rate of *L. tarentolae*. Providing that NMR signals can be reasonably detected even at a low-level of enrichment (~5%), the savings stemming from avoiding the commercially available synthetic medium result in a 3-to-12-fold reduction of the overall costs for sample preparation depending on isotopic label(s) used. The minimal amount of labeled amino acids to be added to complex BHI medium for low-level enrichment can be estimated from general abundances of amino acids in proteins relative to valine (Table I). These amounts correspond to concentrations 3-6 times higher than those recommended for the synthetic medium (Synthetic LEXSY, Jena Bioscience, Germany) (37).

Taken together, the general protocol for low-level isotopic enrichment of over-expressed proteins in *L. tarentolae* has the following basic features: (i) the cell mass is produced by growing *L. tarentolae* in BHI medium; (ii) the cells are transferred into fresh complex BHI medium to remove metabolic end-products inhibitory to the growth and expression, supplemented with labeled amino acid of choice at concentrations 3-6 times higher than those recommended for the synthetic medium; (iii) 1 hour after the transfer, over-expression is induced and allowed to proceed for 24 hours.

Conclusions

The described labeling approach provides an economically efficient alternative to currently established labeling procedure by Nicuale *et al*. (2006) (24). It is expected to find its primary application in NMR studies, where AATS labeling is sufficient and where sample costs are of primary interest for procedures addressing protein folding and conformational homogeneity verifications (often used in the crystallization experiments), monitoring of the effect of post-translational modifications on protein structure and dynamics, screening of protein-drug/ligand interactions or in cell NMR studies. The method is expected to be applicable for labeling with 10 other essential amino acids, namely R, H, M, W, F, S, Y, T, L, and K.

We also expect that a similar approach, *i.e.*, low level enrichment, can be applied to other expression systems, such as the CHO or HEK cells, where the high costs of sample preparation are due to the high price of the synthetic media.

Acknowledgments

This work was supported by Czech Grant Agency (204/08/P585, S60220554), Grant Agency of AS CR (KAN200100801), DAAD, and Ministry of Education of the Czech Republic (MSM6007665801 and 2B06129). Financial support by the Access to Research Infrastructures activity in the 6th Framework Programme of the EC (Contract # RII3-026145, EU-NMR) for conducting the research is gratefully acknowledged. We thank Peter Konik for his assistance with mass spectrometric analysis.

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Date Received: November 30, 2008

Communicated by the Editor Jiri Sponer