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Optimized procedure to generate heavy isotope and selenomethionine labeled proteins for structure determination using *Escherichia coli* based expression systems

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Abstract

Generating sufficient quantities of labeled proteins represents a bottleneck in protein structure determination. A simple protocol for producing heavy isotope as well as selenomethionine (Se-Met) labeled proteins was developed using T7-based *Escherichia coli* expression systems. The protocol is applicable for generation of single, double and triple-labeled proteins (^{15}N , ^{13}C and ^2H) in shaker flask cultures. Label incorporation into the target protein reached 99% and 97% for ^{15}N and ^{13}C , respectively, and 75% of (non-exchangeable) hydrogen for ^2H labeling. The expression yields and final cell densities ($\text{OD}_{600} \sim 16$) were the same as for the production of non-labeled protein. This protocol is also applicable for Se-Met labeling, leading to Se-Met incorporation into the target protein of 70 or 90% using prototrophic or methionine auxotrophic *E. coli* strains, respectively.

Key words: *Escherichia coli*, recombinant protein expression, stable heavy isotope labeling, Selenomethionine labeling, autoinduction, defined medium.

Introduction

Stable heavy isotope (^{15}N , ^{13}C and ^2H) and selenomethionine (Se-Met) labeled proteins are commonly used for protein structure determination through NMR spectroscopy (Gardner and Kay 1998) and X-ray crystallography (Doublie 1997), respectively. The generation of isotope-enriched protein samples is a prerequisite for carrying out structural studies using NMR spectroscopy (Wishart and Sykes 1994; Wishart et al. 1995; Yamazaki et al. 1994). Also, incorporation of Se-Met into proteins considerably improves protein structure studies by X-ray crystallography through phase determination by multi-wavelength anomalous diffraction (Hendrickson et al. 1990; Hendrickson 1991).

As labeling experiments are usually costly, an efficient and simple protocol for generating labeled proteins is of crucial importance to structural biologists. Current protocols for production of heavy isotope and Se-Met labeled proteins using *E. coli* expression systems are mainly based on M9 minimal medium (Doublie 1997; Fiaux et al. 2004; Leiting et al. 1998; Mac et al. 2006; Seo et al. 2009; Zhao et al. 2004) leading to very poor final biomass concentrations (OD600 of 1~2) and, consequently, low yields of labeled proteins. Some improvements have been obtained by optimizing the minimal medium (Jansson et al. 1996) or by using autoinduction medium for the generation of ^{15}N and/or ^{13}C or Se-Met labeled proteins (Studier 2005; Sreenath et al. 2005; Tyler et al. 2005). Moreover, carbon limited fed-batch processes have been developed for production of ^{15}N and ^{13}C (Ross et al. 2004) or Se-Met labeled proteins (Studts and Fox 1999) which lead to higher final biomass concentrations (OD600 of ~6 or ~11 for heavy isotope or Se-Met labeled proteins, respectively) and, thus higher labeled target protein yields. However, the establishment of carbon-limited fed-batch processes usually requires the availability of more sophisticated technical equipment and, more importantly, the availability of dedicated staff. Thus, there is still a great need for protein labeling protocols which are easy to implement and lead to sufficient protein amounts with high label incorporation at acceptable costs.

The production of deuterated (^2H labeled) proteins is even more difficult and very costly with current protocols as multiple adaptation steps of *E. coli* to pure D_2O are required to generate protein with high label content (Paliy et al. 2003). Even after adaptation, cell densities reached in D_2O did not increase above $\text{OD}_{600} \sim 5$ (Romanuka et al. 2009; Paliy et al. 2003). As alternative, cells can be grown in the presence of 75% D_2O to avoid the strong inhibitory effect of pure D_2O on growth and protein production, however, the reduced D_2O content also reduces the deuteration efficiency of the target protein (Marley et al. 2001). Furthermore, for all labeling methods, different protocols are currently employed, all with their unique stock solutions and procedures, making the production of highly labeled proteins tedious, more costly than necessary and most often the bottleneck of protein structure determination.

In this work, we present a unified protocol based on a modular system which can be used to efficiently produce heavy isotope (mono- or multiple-incorporations of ^2H , ^{13}C and ^{15}N) as well as Se-Met labeled proteins with high label content using the T7-based *E. coli* expression system. Special attention is given to the production of deuterated proteins.

Materials and methods

Strains and plasmids

E. coli BL21 (DE3) (Novagen, Germany) and the methionine auxotrophic strain T7 Express Crystal (New England Biolabs, USA) were used. The plasmid pET-15b-hPrP encoding the human prion protein (hPrP, residues 23-231) under control of T7 promoter is described elsewhere (Zahn et al. 1997). Details concerning the plasmids pET-29c-hFGF-2 and pET-28c-His6-GFP encoding human basic fibroblast growth factor (hFGF-2) and His6-tagged green fluorescent protein (GFP) are found elsewhere (Hoffmann et al. 2004 and Li et al. 2011, respectively).

Medium composition and preparation

The composition of the Luria-Bertani (LB) medium was as follows: 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 5 g L⁻¹ NaCl and 15 g L⁻¹ agar added for medium solidification, the final pH was adjusted to pH 7 before autoclaving. The composition and preparation of the defined labeling medium are based on the defined high cell density cultivation medium (Korz et al. 1995) and the defined autoinduction medium (Li et al. 2011). A general scheme for the usage and composition of the defined labeling medium including medium abbreviations are given in Table 1. Detailed instructions for medium preparation are provided in the Supplementary Tables 1 and 2. Appropriate antibiotics were added to the medium for plasmid maintenance (pET-15b-hPrP: 100 mg L⁻¹ ampicillin, pET-29c-hFGF-2 and pET-28c-His6-GFP: 50 mg L⁻¹ kanamycin). D-glucose (U-¹³C₆, 99%), ammonium chloride (¹⁵N, 99%), and deuterium oxide (D, 99.9%) used for labeling experiments were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). L-selenomethionine was purchased from Sigma-Aldrich (Munich, Germany).

Cultivation conditions

Two different cultivation strategies for different labeling purposes were employed (for details refer to Fig. 1). In principle, autoinduction was performed for non-labeled protein production (control run), and for ^{15}N and ^2H labeling. For ^{13}C labeling (or combined with ^2H or ^{15}N labeling) and for selenomethionine labeling, medium exchange was applied (spin down pre-cultures and resuspend cell pellets in labeling medium). Cultivations were carried out in duplicate using 250 ml shake flasks with three baffles containing 25 ml medium at 180 rpm (using a shaker with an amplitude of 5 cm). Mean values are given.

Analysis of cell growth and protein production

Cell growth was monitored by measurement of the absorbance at 600 nm (OD600). For protein production analysis, culture samples were centrifuged at 17,000 g and 4°C for 3 min. After removal of supernatant, cell pellets were stored at -80°C until further analysis. SDS-PAGE analysis was performed in PROTEAN II xi (Bio-Rad, USA) according to standard procedures (Bollag et al. 1996) and instructions from the manufacturer. BugBusterTM Protein Extraction Reagent (Novagen, USA) was used to generate cell extracts and to prepare soluble and insoluble fractions according to the instructions from the manufacturer. After electrophoresis, proteins on SDS-PAGE gels were visualized by colloidal Coomassie G-250 staining (Candiano et al. 2004). Stained wet gels were scanned by ScanMaker 9800XL (Microtek, Taiwan).

Heavy isotope and selenomethionine labeling efficiency determination

The heavy isotope or Se-Met labeling efficiency was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Protein bands were excised manually from the SDS-PAGE gels. After washing, reduction and alkylation, in-gel digestion was carried out by incubation with sequencing grade modified trypsin (Promega, USA). Obtained peptides were extracted and purified with reversed-phased C18 ZipTips (Millipore, USA). Desalted

peptide solutions were mixed with a saturated matrix solution and spotted onto a 384 MTP target and dried at room temperature. A Bruker Ultraflex time-of-flight mass spectrometer (Bruker Daltonics GmbH, Germany) was employed to obtain peptide mass fingerprints. Details of the protocol have been described previously (Lu et al. 2010; Wang et al. 2003).

The mass fingerprint spectra of proteins with different labeling methods were analyzed using the software flexAnalysisTM (Bruker Daltonics GmbH, Germany). The theoretical mass fingerprint spectra of the proteins were generated by PeptideCutter in ExPASy Proteomics Server (<http://www.expasy.org/tools/peptidecutter/>). The formula of each peptide was calculated by ProtParam in ExPASy Proteomics Server (<http://www.expasy.org/tools/protparam.html>). The theoretical peptide isotopic distributions with the different isotope abundances were calculated using IsoPro 3.0 MS/MS software and matched with the experimental mass spectra to determine the isotopic labeling efficiency. The numbers of non-exchangeable and exchangeable hydrogen atoms in the proteins were determined according to the amino acid sequence as described previously (Culebras and Moore 1977). The theoretical isotopic distribution of the peptides with methionine replaced by Se-Met was calculated by IsoPro 3.0 MS/MS software and compared with the experimental mass spectra to help locate the position of peptides containing Se-Met in the experimental mass spectra. The area below the mass spectrum of a peptide containing methionine or Se-Met was integrated. The Se-Met labeling efficiency for each individual peptide was calculated according to the literature (Zhao et al. 2007). Average heavy isotope or Se-Met labeling efficiency of trypsin digested peptides in the experimental MS fingerprint was used to determine the approximate overall labeling efficiency of the protein.

Results

General considerations for developing media for heavy isotope and selenomethionine labeling

Two different types of defined media were developed for generating labeled proteins based on the two methods used for induction of protein production: autoinduction and isopropyl β -D-1-thiogalactopyranoside (IPTG) induction (for details refer to Table 1).

Special attention was given to generate media recipes where labeled substrates of the lowest price can be used. For example, ^{15}N - NH_4Cl is much cheaper than $(^{15}\text{NH}_4)_2\text{HPO}_4$, thus NH_4Cl was used as the sole nitrogen source in labeling medium instead of $(\text{NH}_4)_2\text{HPO}_4$. Usually, ammonium phosphate is the better nitrogen source in defined media as it contributes to a better buffering capacity for pH maintenance and additionally provides the required phosphate for cell growth. When replacing $(\text{NH}_4)_2\text{HPO}_4$ in the medium (e.g. in the Defined Non-inducing Broth [DNB]) by NH_4Cl (e.g. in the Labeling Defined Non-inducing Broth [L-DNB]), the labeling medium contains in addition 60.5 mmol L^{-1} NaCl , which is introduced by pH adjustment using NaOH (instead of NH_4OH). This higher salt content causes a decrease of the specific growth rate by 13% of *E. coli* BL21 (DE3) at 37°C in L-DNB compared to DNB.

If possible, autoinduction was used as a very convenient method for generating labeled proteins as it omits biomass monitoring for the correct timing of inducer addition and places the shift from growth to recombinant protein production under metabolic control of the expression host (Li et al. 2011). Using autoinduction a mixture of glucose, glycerol and lactose is used as carbon substrate. However, the prices of ^{13}C labeled glycerol and lactose are beyond the budget of most research groups and much higher compared to ^{13}C -glucose. Thus, employing the autoinduction method for generating ^{13}C labeled proteins is not advised. In this case, the utilization of ^{13}C labeled glucose as sole carbon source in combination with IPTG induction is the better choice. To reduce labeling costs, cells are pre-grown on non-labeled glucose prior to simultaneous addition of IPTG and ^{13}C -

glucose. For high label incorporation it is recommended to remove unlabelled carbon substrates and potentially toxic by-products such as acetic acid prior to adding IPTG and the labeled substrate. Thus, it is recommended to exchange the medium with fresh medium containing the IPTG and labeled glucose.

Another point to consider is the toxicity of labeled substrates. For example, Se-Met is a very toxic compound and *E. coli* BL21 (DE3) is not able to grow in autoinduction medium with more than 50 mg L⁻¹ Se-Met leading to low label incorporation into the target protein (data not shown). Thus, IPTG induction combined with medium exchange is the best approach for generating Se-Met labeled proteins. A general summary of the specific medium properties for specific labeling purposes is given in Table 1.

Heavy isotope labeling

A set of shake flask experiments was carried out at 37°C for the production of single, double and triple-labeled (¹⁵N, ¹³C and ²H) hPrP using *E. coli* BL21 (DE3) as expression host (details for production are given in Fig. 1). When labeling was performed in autoinduction medium (only ¹⁵N and/or ²H labeling) or by medium exchange and IPTG induction (¹³C labeling alone or in combination with ¹⁵N and/or ²H labeling), final OD values reached OD₆₀₀ ~16 or ~12, respectively (Fig. 2). The expression level and the final biomass yields were not affected by the different labeling strategies and the target protein reached ~21% of the total cell protein (Fig. 2). Even deuterium labeling did not cause a decrease in the expression level and final biomass yield although cells grew considerably slower in D₂O compared to H₂O. For example, the growth rate of *E. coli* BL21 (DE3) decreased by 60% at 37°C when the medium was prepared with D₂O instead of H₂O. The overall labeling efficiency for the different isotopes and isotope combinations was determined from five peptides which cover 38% of the hPrP protein sequence (Table 2). Details of the isotopic distribution are shown in the mass spectrum of the most representative peptide (Fig. 3). For ¹⁵N

labeling, 99% labeling was achieved using autoinduction. For ^{13}C labeling, 97% labeling was achieved with medium exchange and IPTG induction. This slight reduction in ^{13}C labeling efficiency was caused, because cells were grown on non-labeled glucose before they were transferred into fresh labeling medium containing IPTG and ^{13}C glucose. However, this overall high label content also shows that the turnover of ^{12}C -carbon from cells pre-grown on ^{12}C glucose to the newly synthesized target protein was lower than 4%.

For mono ^2H or combined ^2H and ^{15}N labeling, cells were grown in the presence of more than 98% D_2O using autoinduction medium. The overall labeling efficiency for deuterium was determined as 58% (Table 2). This seemingly low label incorporation was obtained because all analytical procedures after cell harvest were performed in H_2O instead of D_2O (e.g. SDS-PAGE, sample preparation for MALDI-TOF analysis). Deuterium at exchangeable hydrogen positions in deuterated proteins (peptides) will exchange to protium (^1H) in the presence of excess H_2O . Thus, the obtained overall labeling efficiency of deuterium of 58% needs to be corrected to obtain the true labeling efficiency at non-exchangeable hydrogen positions (identical to the true labeling efficiency of all hydrogen directly after cell harvest before further sample processing). These corrections revealed a true labeling efficiency for deuterium of 75%. This lower labeling efficiency for deuterium compared to the other heavy isotopes was caused by the utilization of non-deuterated substrates (e.g. carbon substrates) which are incorporated into the biomass and target protein.

For double- and triple-labeling, the labeling efficiency for each isotope was the same as for mono-labeling. However, for combined ^{13}C and ^{15}N labeling, cells should be pre-grown on $^{15}\text{N}\text{-NH}_4\text{Cl}$ before they are transferred to fresh labeling medium containing IPTG, $^{15}\text{N}\text{-NH}_4\text{Cl}$ and ^{13}C glucose, otherwise the ^{15}N labeling efficiency will decrease to 95%. The turnover of N-nitrogen from cells pre-grown on non-labeled nitrogen to the newly synthesized target protein is higher than the C-carbon turnover as nitrogen is always recycled and carbon partially lost as carbon dioxide.

The general applicability of this labeling approach was tested for the production of two more proteins using T7 based expression systems. Moreover, proteins were chosen which can be

produced as soluble protein but also in form of inclusion bodies to examine if label incorporation is different in soluble and aggregated proteins (Fig. 4). The results of these studies revealed that the labeling efficiency was independent of the protein being produced and, moreover, identical in both the soluble and aggregated fraction (Table 3 and Fig. 5).

Selenomethionine labeling

Se-Met is toxic for *E. coli*, thus a similar labeling strategy with medium exchange was chosen as used for generating ^{13}C labeled proteins. Cells were first grown on non-inducing medium which was supplemented with 250 mg L^{-1} methionine to suppress the methionine biosynthesis pathway. Thereafter, cells were collected by centrifugation and resuspended in fresh medium containing IPTG, glucose as carbon substrate and Se-Met (experimental details are given in Fig. 1). Production of hPrP was studied in media supplemented with different Se-Met concentrations using the methionine prototrophic strain *E. coli* BL21 (DE3) and the auxotrophic strain T7 Express Crystal (Fig. 6). The final cell densities and expression yields ($\text{OD}_{600} \sim 10$ and 22% hPrP of total cell protein) was independent on the Se-Met concentration ($25 - 200\text{ mg L}^{-1}$) for *E. coli* BL21 (DE3). However, for the auxotrophic strain T7 Express Crystal a strong influence of the Se-Met concentration on the final biomass yield and expression level was observed (Fig. 6). For this strain, the final biomass and the target protein expression level increased with increasing Se-Met concentration (up to 150 mg L^{-1}) reaching a maximum of $\text{OD}_{600} \sim 20$ and 17% hPrP of total cell protein, respectively. Compared to the methionine prototrophic strain *E. coli* BL21 (DE3) the methionine auxotrophic strain (T7 Express Crystal) exhibited a higher tolerance towards the toxic Se-Met by reaching higher cell densities, however the target protein expression level was lower. The overall Se-Met labeling efficiency of hPrP produced in *E. coli* BL21 (DE3) and T7 Express Crystal was determined from three and four peptides, respectively, containing at least five of the nine methionines of hPrP (Table 4). Details of the isotopic distribution are shown in the mass

spectra of two representative peptides (Fig. 7). For *E. coli* BL21 (DE3), the Se-Met labeling efficiency increased from 45% to 70% with increasing Se-Met concentration reaching the maximum efficiency of 70% at 150 or 200 mg L⁻¹ Se-Met. For *E. coli* T7 Express Crystal, higher labeling efficiencies were achieved, increasing from 75% to 90% with increasing Se-Met concentration and reaching the highest efficiency of 90% at 200 mg L⁻¹ Se-Met.

Conclusions

A unified protocol based on a modular system for production of labeled proteins using *E. coli* based expression systems was developed. This protocol can be used to efficiently produce heavy isotope (mono- or multiple-incorporations of ^2H , ^{13}C and ^{15}N) as well as Se-Met labeled proteins with high label content. The labeling efficiency reached 99% and 97% for ^{15}N and ^{13}C -labeling, respectively. The ^{13}C - labeling efficiency can be further increased when cells are pre-cultured on labeled glucose prior to induction of labeled protein production. The labeling efficiency for deuterated proteins was at least 75% with 80% as the theoretical maximal ^2H labeling efficiency when using non-deuterated carbon substrates. Incorporation of 99% deuterium can only be achieved when deuterated carbon substrates are used (Leiting et al. 1998). We also show that double and triple-heavy isotope labeling did not change the labeling efficiency achieved by mono-labeling. Moreover, the labeling efficiency was independent of the target protein and identical for soluble and aggregated proteins. However, for the production of soluble proteins it might be necessary to use lower culture temperatures during labeling. Labeling efficiency for Se-Met incorporation was 70% using the conventional *E. coli* strain BL21 (DE3) or increased to 90% using the methionine auxotrophic strain T7 Express Crystal. Incorporation of other labeled amino acids is possible by employing the appropriate labeled amino acid and the corresponding auxotrophic host strain. The target protein expression level as well as its solubility is characteristic for each protein, however, expression yields and solubility in labeling medium were not different from those obtained in non-labeling medium. Final cell densities and product titers for all labeling purposes were the most efficient so far reaching for deuterated proteins ~4 times higher levels as in previously published protocols.

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Figures captions

Fig. 1 Cultivation strategies for different labeling purposes.

Fig. 2 Heavy isotope labeling of hPrP protein. Cells were grown at 37°C in labeling medium and protein production analyzed by SDS-PAGE. The labeling strategies and final cell densities (OD600) are indicated on top of the figure. The analyzed cell fractions are indicated below (W: whole cell protein. S: soluble part. I: insoluble part of whole cell protein. M: Marker).

Fig. 3 Mass spectra of representative hPrP peptide after heavy isotope labeling. (A) Experimental mass spectra of the YPNQVYYRPVDQYNNQNNFVHDC_(CAM)VNITIK peptide resulting from different heavy isotope labeling strategies (indicated in the right upper corner of each panel). (B) Theoretical mass spectra of the YPNQVYYRPVDQYNNQNNFVHDC_(CAM)VNITIK peptide (Formula: C₁₆₁H₂₃₆N₄₅O₄₉S₁) at different isotope abundances (indicated in the left upper corner of each panel). The Y axis of each panel indicates the relative abundance of MS peaks and the X axis indicates the mass/charge (m/z) ratio. C_(CAM): carbamidomethyl cysteine.

Fig. 4 Heavy isotope labeling of GFP and hFGF-2. Cells were grown at 30°C in labeling medium and protein production analyzed by SDS-PAGE. The labeling strategies and final cell densities (OD600) are indicated on top of the figure. The analyzed cell fractions are indicated below (W: whole cell protein. S: soluble part. I: insoluble part of whole cell protein. M: Marker. N: non-induced negative control)

Fig. 5 Mass spectra of representative peptides of hFGF-2 and GFP after ^2H and ^{15}N double-labeling. (A) Experimental mass spectra of the $\text{C}_{(\text{CAM})}\text{VTDEC}_{(\text{CAM})}\text{FFFER}$ peptide of hFGF-2: (A1) non-labeled hFGF-2, (A2) ^2H and ^{15}N labeled in soluble and (A3) insoluble fraction of hFGF-2. (B) Theoretical mass spectra of the $\text{C}_{(\text{CAM})}\text{VTDEC}_{(\text{CAM})}\text{FFFER}$ peptide (Formula: $\text{C}_{62}\text{H}_{86}\text{N}_{14}\text{O}_{19}\text{O}_2 + \text{C}_4\text{H}_6\text{N}_2\text{O}_2$) of hFGF-2: (B1) non-labeling control and (B2) ^2H and ^{15}N labeled peptide. (C) Experimental mass spectra of the LEYNYNSHNVYITADKQK peptide of GFP: (C1) non-labeled GFP, (C2) ^2H and ^{15}N labeled in soluble and (C4) insoluble fraction of GFP. (D) Theoretical mass spectra of the LEYNYNSHNVYITADKQK peptide (Formula: $\text{C}_{98}\text{H}_{146}\text{N}_{26}\text{O}_{32}$) of GFP: (D1) non-labeling control and (D2) ^2H and ^{15}N labeled peptide. The Y axis of each panel indicates the relative abundance of MS peaks and the X axis indicates the mass/charge (m/z) ratio. $\text{C}_{(\text{CAM})}$: carbamidomethyl cysteine.

Fig. 6 Selenomethionine labeling of hPrP protein. Cells were grown at 37°C in labeling medium and the whole cell protein analyzed by SDS-PAGE. The host strain and the final cell densities (OD_{600}) are indicated on top of the figure. Lane numbers are indicated below. M: marker; lanes 1-7: hPrP produced in T7 Express Crystal (methionine auxotroph), lane 1: positive control with 200 mg L^{-1} methionine, lane 2: negative control without methionine and Se-Met, lane 3: 25 mg L^{-1} Se-Met, lane 4: 50 mg L^{-1} Se-Met, lane 5: 100 mg L^{-1} Se-Met, lane 6: 150 mg L^{-1} Se-Met, lane 7: 200 mg L^{-1} Se-Met; lanes 8-13: hPrP produced in *E.coli* BL21 (DE3), lane 8: 25 mg L^{-1} Se-Met, lane 9: 50 mg L^{-1} Se-Met, lane 10: 100 mg L^{-1} Se-Met, lane 11: 150 mg L^{-1} Se-Met, lane 12: 200 mg L^{-1} Se-Met, lane 13: positive control with 200 mg L^{-1} methionine.

Fig. 7 Mass spectra of two representative hPrP peptides after Se-Met labeling. (A) Experimental mass spectra of T₁: “VVEQMC_(CAM)TTQYQK” and T₂: “PMMHFGNDWEDR” peptides. (B) Theoretical mass spectra of the T₁ peptide: (B1) VVEQMC_(CAM)TTQYQK (m/z: 1515.7, T₁[Met]) and (B2) VVEQM_(Seleno)C_(CAM)TTQYQK (m/z: 1562.7, T₁[Se-Met]). Theoretical mass spectra of the T₂ peptide: (B3) PMMHFGNDWEDR (m/z: 1535.7, T₂[Met]₂) and (B4) PM_(Seleno)M_(Seleno)HFGNDWEDR (m/z: 1628.6, T₂[Se-Met]₂). The host strain and Se-Met concentrations in the labeling medium are indicated in the right upper corner of each panel. The Y axis of each panel indicates the relative abundance of MS peaks and the X indicates axis the mass/charge (m/z) ratio. Met: methionine, Met_{ox}: oxidized methionine, Se-Met and M_(Seleno): Selenomethionine, C_(CAM): carbamidomethyl cysteine.

Tables

Table 1 General scheme for the usage and composition of the defined labeling medium

Name of medium	Solvent	General usage	Induction method	Carbon(s) (g L ⁻¹)	Nitrogen (g L ⁻¹)	KH ₂ PO ₄ (g L ⁻¹)
S-DAB (HNC)	H ₂ O	Non-labeled protein production	Auto-induction	2.9 glucose 11.1 glycerol 7.6 lactose	4.0 (NH ₄) ₂ HPO ₄	13.3
S-DAB	H ₂ O	Non-labeled protein production				
DNB	H ₂ O	Pre-cultures and IPTG induction	IPTG	10.9 glucose		
SL-DAB	H ₂ O	For ¹⁵ N Labeling	Auto-induction	2.9 glucose 11.1 glycerol 7.6 lactose	3.2 ¹⁵ N-NH ₄ Cl	17.4
SL-DAB (in D ₂ O)	D ₂ O	For ² H and ¹⁵ N* labeling				
rL-DNB	H ₂ O	For ¹³ C and ¹⁵ N* labeling	IPTG	6.5 ¹³ C-glucose	2.6 ¹⁵ N-NH ₄ Cl	
rL-DNB (in D ₂ O)	D ₂ O	For ² H, ¹³ C and ¹⁵ N* labeling				
L-DNB (in D ₂ O)	D ₂ O	Pre-culture of ¹⁵ N*, ¹³ C and ² H labeling	IPTG	10.9 glucose	3.2 ¹⁵ N-NH ₄ Cl	
L-DNB	H ₂ O	Pre-cultures and Se-Met labeling			3.2 NH ₄ Cl	

S-DAB (HNC): Smart-Defined Autoinduction Broth (High Nitrogen Content) with pH adjustment by NH₄OH; S-DAB: Smart-Defined Autoinduction Broth; DNB: Defined Non-inducing Broth; SL-DAB: Smart Labeling-Defined Autoinduction Broth, rL-DNB: reduced Labeling-Defined Non-inducing Broth (with reduced concentrations of carbon and nitrogen substrates), L-DNB: Labeling-Defined Non-inducing Broth. For selenomethionine labeling, the pre-culture and main culture were supplemented with 250 mg L⁻¹ methionine and 200 mg L⁻¹ selenomethionine, respectively. ¹⁵N*: the labeling of ¹⁵N is optional. If ¹⁵N labeling is not required, ¹⁵N-NH₄Cl can be replaced by normal NH₄Cl.

Except for the concentrations of the carbon and nitrogen substrates, potassium phosphate and the method of pH adjustment, the concentrations of remaining components were identical and as follows: 1.55 g L⁻¹ Citric acid, 0.59 g L⁻¹ MgSO₄, 100.8 mg L⁻¹ Fe(III) citrate, 2.1 mg L⁻¹ Na₂MoO₄ · 2H₂O, 2.5 mg L⁻¹ CoCl₂ · 6H₂O, 15 mg L⁻¹ MnCl₂ · 4H₂O, 1.5 mg L⁻¹ CuCl₂ · 2H₂O, 3 mg L⁻¹ H₃BO₃, 33.8 mg L⁻¹ Zn(CH₃COO)₂ · 2H₂O, 14.10 mg L⁻¹ Titriplex III. Except for S-DAB (HNC), the final pH of all other media was adjusted to 6.8 by NaOH. Details for medium preparation are given in the supplementary material.

Table 2 Heavy isotope labeling efficiency of hPrP

Peptide fragment in hPrP	¹⁵ N Labeling	¹³ C Labling	² H labeling	² H and ¹⁵ N labeling	² H, ¹⁵ N and ¹³ C labeling			
	Labeling efficiency (%)							
	¹⁵ N	¹³ C	² H	² H	¹⁵ N	² H	¹⁵ N	¹³ C
YPGQGSPGGNR	99	98	54* (75**)	54* (75**)	99	54* (75**)	99	98
YPNQVYYR	99	98	54* (70**)	54* (70**)	99	54* (70**)	99	98
VVEQMCTTQYQK	99	97	59* (77**)	59* (77**)	99	59* (77**)	99	97
HMAGAAAAGAVVGG LGGYMLGSAMSR	99	98	63* (79**)	63* (75**)	99	63* (75**)	99	98
YPNQVYYRPPVDQYN NQNNFVHDCVNITIK	99	97	57* (75**)	57* (75**)	99	57* (75**)	99	97
Overall hPrP (approximate)	99	97	58* (75**)	58* (75**)	99	58* (75**)	99	97

*: labeling efficiency for deuterium as determined by MALDI-TOF MS.

**: corrected as true labeling efficiency at non-exchangeable hydrogen positions.

Table 3 Heavy isotope labeling efficiency of hFGF-2 and GFP

Peptide fragment in hFGF-2	² H and ¹⁵ N labeling efficiency (%)		Peptide fragment in GFP	² H and ¹⁵ N labeling efficiency (%)	
	² H	¹⁵ N		² H	¹⁵ N
YLAMKEDGR	63 [*] (82 ^{**})	99	FEGDTLVNR	61 [*] (80 ^{**})	99
IHPDGRVDGVR	64 [*] (81 ^{**})	99	SAMPEGYVQER	60 [*] (77 ^{**})	99
GVVSIKGVCANR	61 [*] (78 ^{**})	99	AEVKFEGDTLVNR	61 [*] (79 ^{**})	99
YTSWYVALKR	60 [*] (74 ^{**})	99	GIDFKEDGNILGHK	59 [*] (76 ^{**})	99
CVTDECFFFER	59 [*] (75 ^{**})	99	LEYNYNSHNVYITADKQK	58 [*] (75 ^{**})	99
Overall hFGF-2 (approximate)	61[*] (78^{**})	99	Overall GFP (approximate)	60[*] (77^{**})	99

*: labeling efficiency for deuterium as determined by MALDI-TOF MS.

**: corrected as true labeling efficiency at non-exchangeable hydrogen positions.

Table 4 Se-Met labeling efficiency of hPrP in different host strains

Peptide fragment in hPrP	Labeling efficiency in <i>E. coli</i> BL21 (DE3) at different Se-Met concentrations			
	50 mg L ⁻¹	100 mg L ⁻¹	150 mg L ⁻¹	200 mg L ⁻¹
VVEQM <u>C</u> TTQYQK	33%	50%	75%	70%
PMMHFGNDWEDRYR	56%	55%	77%	74%
H <u>M</u> AGAAAAGAVVGGL GGY <u>M</u> LGSAM <u>S</u> R	43%	62%	67%	70%
Overall hPrP (approximate)	45%	55%	70%	70%
Peptide fragment in hPrP	Labeling efficiency in T7 Express Crystal at different Se-Met concentrations			
	50 mg L ⁻¹	100 mg L ⁻¹	150 mg L ⁻¹	200 mg L ⁻¹
YYERN <u>M</u> NR	74%	68%	88%	95%
VVEQM <u>C</u> TTQYQK	74%	83%	81%	89%
PMMHFGNDWEDR	72%	69%	83%	86%
GENFTETDIK <u>I</u> MER	86%	91%	90%	92%
Overall hPrP (approximate)	75%	75%	85%	90%

Fig 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

Applied Microbiology and Biotechnology

Supplementary Material

Optimized procedure to generate heavy isotope and selenomethionine labeled proteins for structure determination using *Escherichia coli* based expression systems

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Medium preparation for heavy isotope and selenomethionine labeling

Supplementary Table 1 Media preparation (For 1 Liter)

Groups		No.	Name of media	S-DAB (HNC) [#]	S-DAB [#]	DNB [#]	SL-DAB ^{##}	SL-DAB (in D ₂ O) ^{##}	rL-DNB ^{##}	rL-DNB (in D ₂ O) ^{##}	L-DNB (in D ₂ O) ^{##}	L-DNB [#]
			General usage or of the medium	No labeled production with good oxygen supply	No labeled production with intermediate oxygen supply	Pre-cultures and IPTG induction	¹⁵ N labeling	² H and ¹⁵ N* labeling	¹³ C and ¹⁵ N* labeling	² H, ¹³ C and ¹⁵ N* labeling	Pre-culture for ¹⁵ N*, ¹³ C and ² H labeling	Pre-cultures and Se-Met labeling
			Inducer	Lactose	Lactose	IPTG	Lactose	Lactose	IPTG	IPTG	IPTG	IPTG
			Volume of stock solution	ml	ml	ml	ml	ml	ml	ml	ml	ml
A group	Magnesium	F1	50 X MgSO ₄ stock solution (H ₂ O)	20	20	20	20		20			20
		F2	50 X MgSO ₄ stock solution (D ₂ O)					20		20	20	
	Carbon source	A1	4 X 2.94-11.07-7.6 carbon source stock solution (H ₂ O)	250	250		250					
		A2	4 X 2.94-11.07-7.6 carbon source stock solution (D ₂ O)					250				
		A3	20 X Glucose stock solution (H ₂ O)			50						50
		A4	20 X Glucose stock solution (D ₂ O)								50	
		A5	¹³ C labeled Glucose stock solution (H ₂ O)						50			
		A6	¹³ C labeled Glucose stock solution (D ₂ O)							50		
	Trace	G1	5000 X Na ₂ MoO ₄ .H ₂ O stock solution (H ₂ O)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
B group	elements	H1	2000 X trace element stock solution (H ₂ O)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Ammonium	B1	50 X (NH ₄) ₂ HPO ₄ stock solution (H ₂ O)	20	20	20						
		B2	50 X NH ₄ Cl stock solution (H ₂ O)						16*			20
		B3	50 X ¹⁵ N labeled NH ₄ Cl stock solution (H ₂ O)				20					
		B4	50 X ¹⁵ N labeled NH ₄ Cl stock solution (D ₂ O)					20*		16*	20*	
		B5	50 X NH ₄ Cl stock solution (D ₂ O)									
	Phosphate and other salts	C1	10 X KH ₂ PO ₄ (High concentration) stock solution (H ₂ O)				100		100			100
		C2	10 X KH ₂ PO ₄ (High concentration) stock solution (D ₂ O)					100		100	100	
		C3	10 X KH ₂ PO ₄ (Low concentration) stock solution (H ₂ O)	100	100	100						
		D1	50 X Citric acid stock solution (H ₂ O)	20	20	20	20		20			20
		D2	50 X Citric acid stock solution (D ₂ O)					20		20	20	
		E1	50 X Fe(III) citrate stock solution (H ₂ O)	20	20	20	20		20			20
		E2	50 X Fe(III) citrate stock solution (D ₂ O)					20		20	20	
	pH adjusting	I1	25 X NH ₄ OH stock solution (H ₂ O) (1:10)	45~50								
		J1	50 X 5M NaOH stock solution (H ₂ O)		13.5	13.5	20		20			20
		J2	50 X 5M NaOH stock solution (D ₂ O)					20		20	20	
	Solvent	H1	Milli-Q water (H ₂ O)	520	556	756	550		754			750
		H2	Deuterium oxide (D ₂ O)					550		754	750	

A group: Mix magnesium and carbon source solution, sterilize. After adding 5000 X Na₂MoO₄.H₂O stock solution, the solution (A group) can be kept for long term storage at room temperature. **B group:** Mix ammonium, phosphate and other salts and add solvent, adjust to pH 6.8, sterilize. After adding 2000 X trace element stock solution, the solution (B group) can be kept for long term storage at room temperature. #:autoclave (or sterilize by filtration); ##: sterilize by filtration.*: the labeling of ¹⁵N is optional. If ¹⁵N incorporation is not required, ¹⁵N-NH₄Cl in the medium can be replaced by normal NH₄Cl.

Medium preparation for heavy isotope and selenomethionine labeling

Supplementary Table 2 Stock solution preparation

No.	Name	Working concentration	Stock solution preparation	Sterilization method
F1	50 X MgSO ₄ stock solution (H ₂ O)	0.586 g L ⁻¹ MgSO ₄	Add 30 g MgSO ₄ ·7H ₂ O (MW: 246.47) to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
F2	50 X MgSO ₄ stock solution (D ₂ O)		Add 3 g MgSO ₄ ·7H ₂ O (MW: 246.47) [or 1.47 g MgSO ₄ (MW: 120.37)] to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
A1	4 X 2.94-11.07-7.6 carbon source stock solution (H ₂ O)	2.94 g L ⁻¹ glucose 11.07 g L ⁻¹ glycerol 7.6 g L ⁻¹ lactose	Add 12.92 g glucose·H ₂ O (MW: 198.17), 44.28 g glycerol (MW: 92.09) and 32 g lactose·H ₂ O (MW: 360.31) to a 1 L graduated cylinder. Bring final volume to 1 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
A2	4 X 2.94-11.07-7.6 carbon source stock solution (D ₂ O)		Add 1.292 g glucose·H ₂ O (MW: 198.17), 4.428 g glycerol (MW: 92.09) and 3.2 g lactose·H ₂ O (MW: 360.31) [or 1.176 g glucose (MW: 180.16), 4.428 g glycerol (MW: 92.09) and 3.04 g lactose (MW: 342.30)] to a 100 ml graduated cylinder. Bring final volume to 100 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
A3	20 X Glucose stock solution (H ₂ O)	10.91 g L ⁻¹ glucose	Add 240 g glucose·H ₂ O (MW: 198.17) to a 1 L graduated cylinder. Bring final volume to 1 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
A4	20 X Glucose stock solution (D ₂ O)		Add 24 g glucose·H ₂ O (MW: 198.17) [or 21.82 glucose (MW: 180.16)] to a 100 ml graduated cylinder. Bring final volume to 100 ml with deuterium monoxide (D ₂ O).	121°C autoclave or filtration (0.2 µm)
A5	¹³ C labeled Glucose stock solution (H ₂ O)	6.55 g L ⁻¹ ¹³ C-glucose	Add 6.546 g ¹³ C-glucose to a 50 ml graduated cylinder. Bring final volume to 50 ml with water (Milli-Q or similar quality).	Filtration (0.2 µm)
A6	¹³ C labeled Glucose stock solution (D ₂ O)		Add 6.546 g ¹³ C-glucose to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
G1	5000 X Na ₂ MoO ₄ ·H ₂ O stock solution (H ₂ O)	2.1 mg L ⁻¹ Na ₂ MoO ₄ ·2H ₂ O	Add 525 mg of Na ₂ MoO ₄ ·2H ₂ O (MW: 241.95) to a 50 ml graduated cylinder. Bring final volume to 50 ml with water (Milli-Q or similar quality).	Filtration (0.2 µm)
H1	2000 X trace element stock solution (H ₂ O)	2.5 mg L ⁻¹ CoCl ₂ ·6H ₂ O 15 mg L ⁻¹ MnCl ₂ ·4H ₂ O 1.5 mg L ⁻¹ CuCl ₂ ·2H ₂ O 3 mg L ⁻¹ H ₃ BO ₃ 33.8 mg L ⁻¹ Zn(CH ₃ COO) ₂ ·2H ₂ O 14.10 mg L ⁻¹ Titriplex III	Add 0.5 g of CoCl ₂ ·6H ₂ O (MW: 237.93), 3 g of MnCl ₂ ·4H ₂ O (MW: 197.9), 0.3 g of CuCl ₂ ·2H ₂ O (MW: 170.48), 0.6 g of H ₃ BO ₃ (MW: 61.83), 6.76 g of Zn(CH ₃ COO) ₂ ·2H ₂ O (MW: 219.49), 2.82 g of Titriplex III (MW: 372.24) to a 100 ml graduated cylinder. Bring final volume to 100 ml with Milli-Q water (or similar quality).	Filtration (0.2 µm)
B1	50 X (NH ₄) ₂ HPO ₄ stock solution (H ₂ O)	4 g L ⁻¹ (NH ₄) ₂ HPO ₄	Add 100 g (NH ₄) ₂ HPO ₄ (MW: 132.06) to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
B2	50 X NH ₄ Cl stock solution (H ₂ O)	3.24 g L ⁻¹ NH ₄ Cl	Add 81 g NH ₄ Cl (MW: 53.49) to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
B3	50 X ¹⁵ N labeled NH ₄ Cl stock solution (H ₂ O)	3.24 g L ⁻¹ ¹⁵ N-NH ₄ Cl	Add 8.1 g ¹⁵ N-NH ₄ Cl to a 50 ml graduated cylinder. Bring final volume to 50 ml with water (Milli-Q or similar quality).	Filtration (0.2 µm)
B4	50 X ¹⁵ N labeled NH ₄ Cl stock solution (D ₂ O)		Add 8.1 g ¹⁵ N-NH ₄ Cl to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
B5	50 X NH ₄ Cl stock solution (D ₂ O)	3.24 g L ⁻¹ NH ₄ Cl	Add 8.1 g NH ₄ Cl (MW: 53.49) to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
C1	10 X KH ₂ PO ₄ (High concentration) stock solution (H ₂ O)	17.42 g L ⁻¹ KH ₂ PO ₄	Add 174.2 g KH ₂ PO ₄ (MW: 136.09) to a 1 L graduated cylinder. Bring final volume to 1 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
C2	10 X KH ₂ PO ₄ (High concentration) stock solution (D ₂ O)		Add 17.42 g KH ₂ PO ₄ (MW: 136.09) to a 100 ml graduated cylinder. Bring final volume to 100 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
C3	10 X KH ₂ PO ₄ (Low concentration) stock solution (H ₂ O)	13.3 g L ⁻¹ KH ₂ PO ₄	Add 133 g KH ₂ PO ₄ (MW: 136.09) to a 1 L graduated cylinder. Bring final volume to 1 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
D1	50 X Citric acid stock solution (H ₂ O)	1.55 g L ⁻¹ Citric acid	Add 42.5 g Citric acid·H ₂ O (MW: 210.14) to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
D2	50 X Citric acid stock solution (D ₂ O)		Add 4.25 g Citric acid·H ₂ O (MW: 210.14) [or 3.886 g Citric acid (MW: 192.12)] to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
E1	50 X Fe(III) citrate stock solution (H ₂ O)	100.8 mg L ⁻¹ Fe(III) citrate	Add 2.52 g Fe(III) citrate (MW: 244.94) to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	121°C autoclave or filtration (0.2 µm)
E2	50 X Fe(III) citrate stock solution (D ₂ O)		Add 0.252 g Fe(III) citrate (MW: 244.94) to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	Filtration (0.2 µm)
I1	25 X NH ₄ OH stock solution (H ₂ O) (1:10)	To bring final pH to 6.8	Add 50 ml 30–33 % Ammonium hydroxide to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	
J1	50 X 5M NaOH stock solution (H ₂ O)		Add 100 g NaOH (MW: 40) to a 0.5 L graduated cylinder. Bring final volume to 0.5 L with water (Milli-Q or similar quality).	
J2	50 X 5M NaOH stock solution (D ₂ O)		Add 10 g NaOH (MW: 40) to a 50 ml graduated cylinder. Bring final volume to 50 ml with deuterium monoxide (D ₂ O).	