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ARTICLE

An economical method for producing stable-isotope labeled proteins by the *E. coli* cell-free system

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Abstract Improvement of the cell-free protein synthesis system (CF) over the past decade have made it one of the most powerful protein production methods. The CF approach is especially useful for stable-isotope (SI) labeling of proteins for NMR analysis. However, it is less popular than expected, partly because the SI-labeled amino acids used for SI labeling by the CF are too expensive. In the present study, we developed a simple and inexpensive method for producing an SI-labeled protein using *Escherichia coli* cell extract-based CF. This method takes advantage of endogenous metabolic conversions to generate SI-labeled asparagine, glutamine, cysteine, and tryptophan, which are much more expensive than the other 16 kinds of SI-labeled amino acids, from inexpensive sources, such as SI-labeled algal amino acid mixture, SI-labeled indole, and sodium sulfide, during the CF reaction. As compared with the conventional method employing 20 kinds of SI-labeled amino acids, highly enriched uniform SI-labeling with similar labeling efficiency was achieved at a greatly reduced cost with the newly developed method. Therefore, our method solves the cost problem of the SI labeling of proteins using the CF.

Keywords Cell-free protein synthesis · In vitro translation · Stable-isotope labeling · Metabolic conversion

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Introduction

Recent technological advances have made it possible to produce a wide variety of eukaryotic and prokaryotic proteins in large quantities by using the cell-free protein synthesis system (CF). With respect to stable-isotope (SI) labeling of proteins for NMR analysis, both highly enriched and amino acid-selective labeling have been enabled by many kinds of improvements (Kigawa et al. 1995; Matsuda et al. 2007; Morita et al. 2004; Ozawa et al. 2004; Wu et al. 2006). A comparison of the cell-based and CF-based methods for protein production indicated that the CF had advantages in terms of NMR-based high-throughput sample screening and labor cost (Tyler et al. 2005). Optimization efforts have focused on making CF simpler and more economical (Calhoun and Swartz 2005; Kim et al. 2006; Kim et al. 2007; Liu et al. 2008). Many structures of functional protein domains were determined in large-scale structural genomics projects by using the high-throughput robotic CF and high performance NMR spectroscopy (Aoki et al. 2009; Kigawa et al. 2004; Vinarov et al. 2004; Yabuki et al. 2007; Yokoyama 2003). The CF is now considered as a suitable method for membrane protein production for structure analysis (Shimono et al. 2009; Sobhanifar et al. 2010). The stereo-array isotope labeling (SAIL) method (Kainosho et al. 2006), which

is expected to expand the molecular size limit of NMR spectroscopy, was achieved by using the CF. However, the CF is still not very popular in NMR applications, partly due to the high cost of SI-labeled amino acids.

Generally, uniformly SI-labeled amino acids are produced from an acid hydrolysate of a stable-isotopically enriched algal protein biomass. However, L-asparagine (Asn), L-glutamine (Gln), L-cysteine (Cys), and L-tryptophan (Trp) are sensitive to acid hydrolysis and thus are not present in the hydrolysate (Hansen et al. 1992). These four acid-sensitive amino acids are prepared individually by using more complicated and time-consuming methods, and consequently are much more expensive than the other 16 amino acids contained in the hydrolysate. Instead of using costly purified SI-labeled amino acids, a relatively inexpensive SI-labeled algal amino acid mixture (AAM) can be used to reduce the labeling cost, but the expensive SI-labeled Asn, Gln, Cys, and Trp are still required (Kigawa et al. 1999; Kigawa et al. 2004). Therefore, the cost of SI labeling using the CF strongly depends on that of the SI-labeled, acid-sensitive amino acids.

Escherichia coli is generally capable of producing all 20 kinds of amino acids endogenously. Asn is synthesized from L-aspartate (Asp) and an ammonium ion by asparagine synthetase (EC 6.3.1.1) and Gln is formed from L-glutamate (Glu) and an ammonium ion by glutamine synthetase (EC 6.3.1.2). Cys is synthesized from Ser by a two-step pathway. Serine *O*-acetyltransferase (SAT) (EC 2.3.1.30), the first enzyme in this pathway, catalyzes the formation of *O*-acetyl-L-serine from L-serine (Ser) and acetyl coenzyme A (AcCoA). The second enzyme, cysteine synthase (CSase) (EC 2.5.1.47), catalyzes the formation of Cys from *O*-acetyl-L-serine and a hydrogen sulfide (H_2S). Trp is synthesized from Ser and an indole by the reaction of tryptophan synthase (TSase) (EC 4.2.1.20) or tryptophanase (TNase) (EC 4.1.99.1) (Fig. 1). The activities of these amino acid metabolism enzymes are retained in the *E. coli* cell extract-based CF, causing SI scrambling and dilution, and are suppressed by adding inhibitors (Ozawa et al. 2004) or by using cell extracts prepared from mutant strains deficient in these enzymes (Calhoun and Swartz 2006). These solutions for suppressing amino acid metabolism contribute to the increased labeling efficiency and thus reduce the cost of SI labeling to some extent. In the present study, we positively utilized the amino acid metabolism, which is generally considered as unfavorable for SI labeling, to endogenously generate acid-sensitive amino acids, i.e., Asn, Gln, Cys, and Trp, in the *E. coli* CF reaction. This dramatically reduces the cost of SI labeling using the CF, because the expensive SI-labeled amino acids are derived from inexpensive sources.

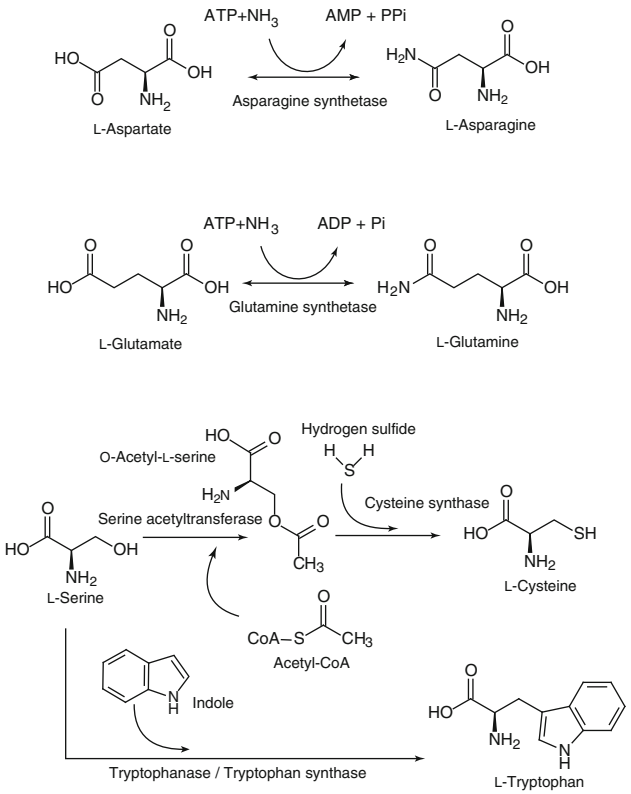

Fig. 1 Biosynthetic pathways of Asn, Gln, Cys, and Trp in *Escherichia coli*

Materials and methods

Cell-free protein synthesis and NMR sample preparation

For chloramphenicol acetyltransferase (CAT) synthesis, the pk7-CAT plasmid (Kim et al. 1996) was used. The CAT productivity was calculated as previously described (Kigawa et al. 2004). For green fluorescence protein (GFP) synthesis, the pGFPS1 plasmid, harboring a gene encoding the mutant protein GFPS1 (Seki et al. 2008), was used. The composition of the *E. coli* cell extract-based CF reaction using D-glutamate was previously described (Matsuda et al. 2007). The batch mode of the CF was performed as described (Kigawa et al. 1999). The dialysis mode of the CF, using the small-scale dialysis unit, and the affinity purification of the product protein were accomplished as described (Matsuda et al. 2007). All of the proteins for NMR measurements were prepared using the dialysis mode method, with 3 mL of internal solution and 30 mL of external solution (Kigawa et al. 2007). Uniformly ^{15}N-labeled Ras(Y32 W) protein (Matsuda et al. 2007; Yamasaki et al. 1994) (BMRB: 10051) (Ras(Y32 W)/20AAM) was synthesized with 3 mg/mL of uniformly ^{15}N-labeled algal AAM (Isotec, Ohio, USA), which

contained 16 kinds of amino acids, except for Asn, Gln, Cys, and Trp, 1 mM each of ^{15}N-Asn (Isotec), ^{15}N-Gln (Isotec), ^{15}N-Cys (Taiyo Nippon Sanso, Tokyo, Japan), and 0.3 mM ^{15}N-Trp (Taiyo Nippon Sanso) as the standard condition. The ^{15}N-labeled Ras(Y32 W)/16AAM was synthesized with 3 mg/mL of ^{15}N-labeled algal AAM, 27 mM ^{15}N-labeled ammonium acetate (Isotec), 0.3 mM ^{15}N-labeled indole (Cambridge Isotope Laboratories, Inc., CIL, Massachusetts, USA), 1 mM sodium sulfide (Na_2S) nonahydrate (Wako Pure Chemicals, Osaka, Japan), which was converted into H_2S in the CF reaction, and 0.2 mM pyridoxal 5′-phosphate (PLP) (Wako Pure Chemicals), which was added for the enhancement of PLP-requiring enzymes, and 0.1 mM acetyl coenzyme A (AcCoA). Indole, a precursor of Trp, was dissolved in 50% ethanol to prepare a 30 mM stock solution, and Na_2S, a precursor of Cys, was freshly dissolved in water before use. Stock solutions of the inhibitors, amino-oxyacetate (AOA: Wako Pure Chemicals) (John et al. 1978; Morita et al. 2004), L-methionine sulfoximine (MS: Nakalai Tesuque, Kyoto, Japan) (Manning et al. 1969), *S*-methyl-L-cysteine sulfoximine (SMCS), synthesized as described previously (Koizumi et al. 1999), and D-malate (Wako Pure Chemicals) (Falzone et al. 1988), were prepared by dissolving them in water at a concentration of 200 mM each. Uniformly ^{15}N-labeled ubiquitin-associated (UBA) domains (Leu655-Ser715 of ubiquitin carboxyl-terminal hydrolase 5 (SWISS-PROT: P45974, PDB: 2DAG, BMRB: 11173), UBA/20AAM and UBA/16AAM, were synthesized using the same conditions as for Ras(32 W)/20AAM and Ras(32 W)/16AAM, respectively. The side-chain selectively ^{15}N-labeled UBA domain was synthesized with 1.5 mM each of 18 non-labeled amino acids (the usual 20 except for Gln and Asn) and 27 mM ^{15}N-labeled ammonium acetate.

Amino acid composition analysis in the cell-free protein synthesis reaction

A 20 μL aliquot of the batch mode of the CF reaction without template DNA was mixed with 20 μL of 5% trichloroacetic acid, and then centrifuged for 10 min at 12,000×*g* to remove the precipitated proteins, as described (Calhoun and Swartz 2006). A 10 μL aliquot of the resultant supernatant was mixed with 70 μL of borate buffer and 20 μL of the derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, from the AccQ-Tag kit (Waters, Massachusetts, USA), and the solution was incubated for 10 min at 55°C in an aluminum block heater. The analyte was applied to a UPLC amino acids analysis system with an AccQ-Tag Ultra Column (2.1 × 100 mm), and was analyzed using the AccQ-Tag method (Waters), in order to investigate amino acid fluctuation during the CF reaction.

HSQC spectra

Labeled Ras(Y32 W) proteins were concentrated to 0.5 mM in 20 mM sodium phosphate buffer (pH 6.5), containing 100 mM NaCl, 5 mM $MgCl_2$, 5 mM *d*-DTT, 0.01% NaN_3, and 10% D_2O. Labeled UBA domains were concentrated to 0.5 mM in 20 mM *d*-Tris–HCl (pH 7.0), containing 100 mM NaCl, 1 mM *d*-DTT, 0.02% NaN_3, and 10% D_2O. Two-dimensional ^{1}H-^{15}N HSQC spectra (Kay et al. 1992) were measured at 25°C on AVANCE 600 MHz or 700 MHz spectrometers equipped with a CryoProbe (Bruker BioSpin, Karlsruhe, Germany). Data were processed using TopSpin 2.1 (Bruker BioSpin).

Results and discussion

CF reaction with a reduced set of amino acids

The CAT protein production reactions using the dialysis mode of the CF, with 1.5 mM each of 20 kinds of amino acids except Asn (20AAM-Asn) (Fig. 2a, lane 1) and 20 kinds of amino acids except Gln (20AAM-Gln) (Fig. 2a, lane 3), were almost comparable to that performed with 1.5 mM each of 20 kinds of amino acids (20AAM) (Fig. 2a, lane 13). In contrast, the CAT protein production reactions with 20AAM-Asn and 20AAM-Gln were almost completely inhibited by adding 20 mM SMCS, a inhibitor of asparagine synthetase (Fig. 2a, lane 2), and 0.5 mM MS, a inhibitor of glutamine synthetase (Fig. 2a, lane 4), respectively. These results indicated that Asn and Gln can be generated from Asp by asparagine synthetase and Glu by glutamine synthetase in the CF reaction mixture, respectively. Very little CAT protein was synthesized with 1.5 mM each of 20 kinds of amino acids except Cys (AAM-Cys) (Fig. 2a, lane 5), whereas the addition of 1 mM Na_2S increased the yield of CAT to more than half of that achieved with 20AAM (Fig. 2a, lane 6). In addition, the CAT production with 20AAM-Cys was further enhanced to the level achieved with 20AAM by adding 1 mM Na_2S, 0.2 mM PLP, and 0.1 mM AcCoA (Fig. 2a, lane 7). The increase in the production was reduced by half by the addition of 20 mM of AOA (Fig. 2a, lane 8), while other inhibitors of SAT and CSase, such as 1 mM $ZnCl_2$ and 1 mM $CuSO_4$, inhibited the CF reaction itself (data not shown). These results indicated that Cys can be generated from its precursors, AcCoA, H_2S, and Ser, by SAT and the CSase cascade in the CF reaction. The CAT production with 20 kinds of amino acids except Trp (20AAM-Trp) (Fig. 2a, lane 9) was dramatically enhanced by adding 0.3 mM indole (Fig 2a, lane 10) and the production was further increased to be equal to that with 20AAM (Fig. 2a, lane 11) by additional supplementation with 0.2 mM PLP.

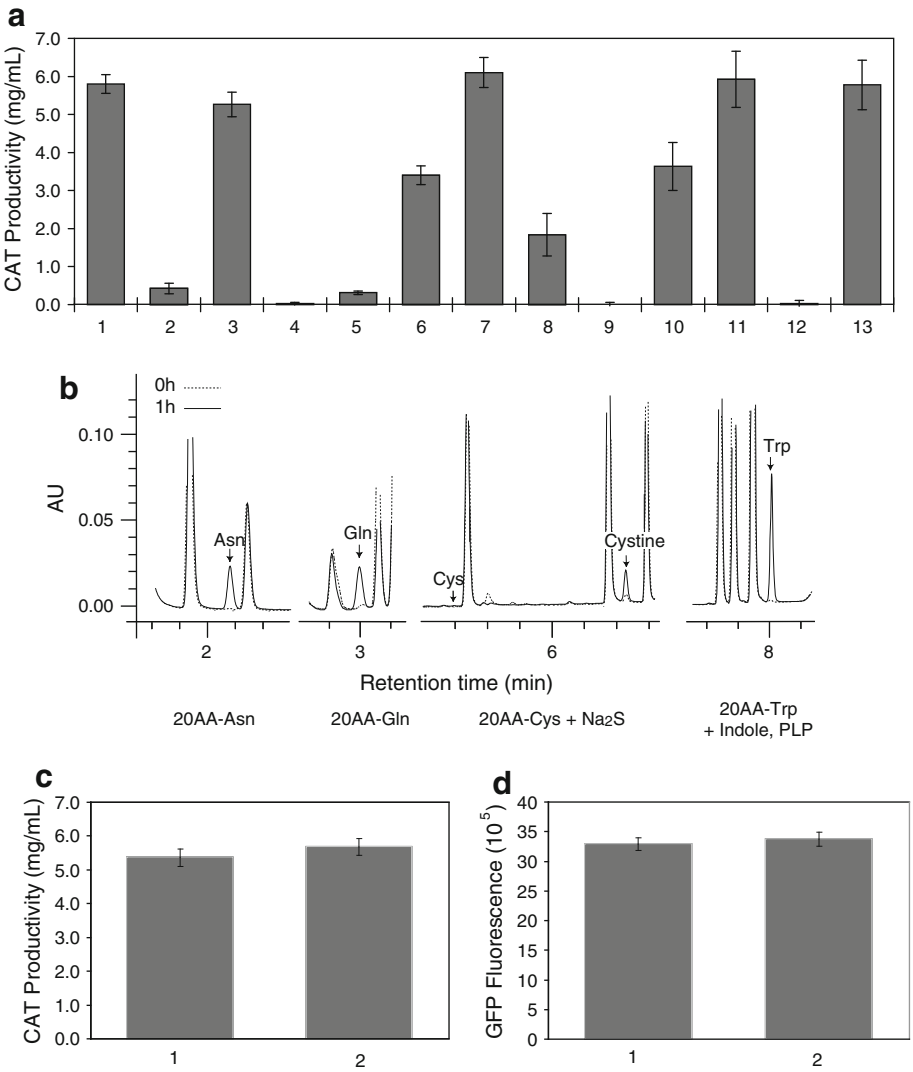

Fig. 2 Cell-free protein synthesis with a reduced set of amino acids. **a** CAT protein production using the dialysis mode of the CF with 20AAM-Asn (*lane 1*), 20AAM-Asn and SMCS (*lane 2*), 20AAM-Gln (*lane 3*), 20AAM-Gln and MS (*lane 4*), 20AAM-Cys (*lane 5*), 20AAM-Cys and Na_2S (*lane 6*), 20AAM-Cys, Na_2S, AcCoA, and PLP (*lane 7*), 20AAM-Cys, Na_2S, and AOA (*lane 8*), 20AAM-Trp (*lane 9*), 20AAM-Trp and indole (*lane 10*), 20AAM-Trp, indole, and PLP (*lane 11*), 20AAM-Trp, indole, and AOA (*lane 12*), and 20AAM (*lane 13*). **b** UPLC amino acid analysis with AccQ-Tag of the reaction solution [before (*dotted line*) and after 1 h (*solid line*) incubations at 37°C] of the batch mode of the CF with 1.5 mM 20AAM-Asn, 1.5 mM 20AAM-Gln, 1.5 mM 20AAM-Cys and Na_2S, and 20AAM-Trp, indole, and PLP, respectively. **c, d** CAT protein (**c**) and GFP protein (**d**) production using the dialysis mode of the CF with 3 mg/mL of ^{15}N-labeled AAM, 0.3 mM ^{15}N-indole, 1 mM Na_2S, 0.2 mM PLP, 0.1 mM AcCoA, and 27 mM ^{15}N-labeled ammonium acetate (*lane 1*), and with 3 mg/mL of ^{15}N-labeled AAM and 1 mM each of Asn, Gln, Cys, and 0.3 mM Trp (*lane 2*), respectively

This enhancement was totally inhibited by adding 20 mM AOA (Fig 2a, lane 12). These results indicated that Trp can be generated from indole and Ser by TSase or TNase in the CF reaction.

The metabolic conversion of amino acids in the CF reaction was further investigated using the UPLC amino acids analysis system with AccQ-Tag, which could analyze the composition of the 20 kinds of amino acids within 10 min. Asn and Gln were detected after a 1 h incubation at 37°C, in the batch mode of the CF reaction solution lacking template DNA and including 1.5 mM 20AAM-Asn and 20AAM-Gln, respectively (Fig. 2b). Similarly, Trp was detected in the reaction solution with 1.5 mM 20AAM-Trp, 0.3 mM indole, and 0.2 mM PLP. On the other hand, only a small amount of Cys was detected in the reaction solution with 1.5 mM 20AAM-Cys, 1 mM Na_2S, and 0.1 mM AcCoA (Fig. 2b), probably because the amount of Cys in the CF reaction solution was maintained at a low level by the feedback regulation of SAT by Cys (Kredich and Tomkins 1966; Mino et al. 1999). These results indicate that Asn, Gln,

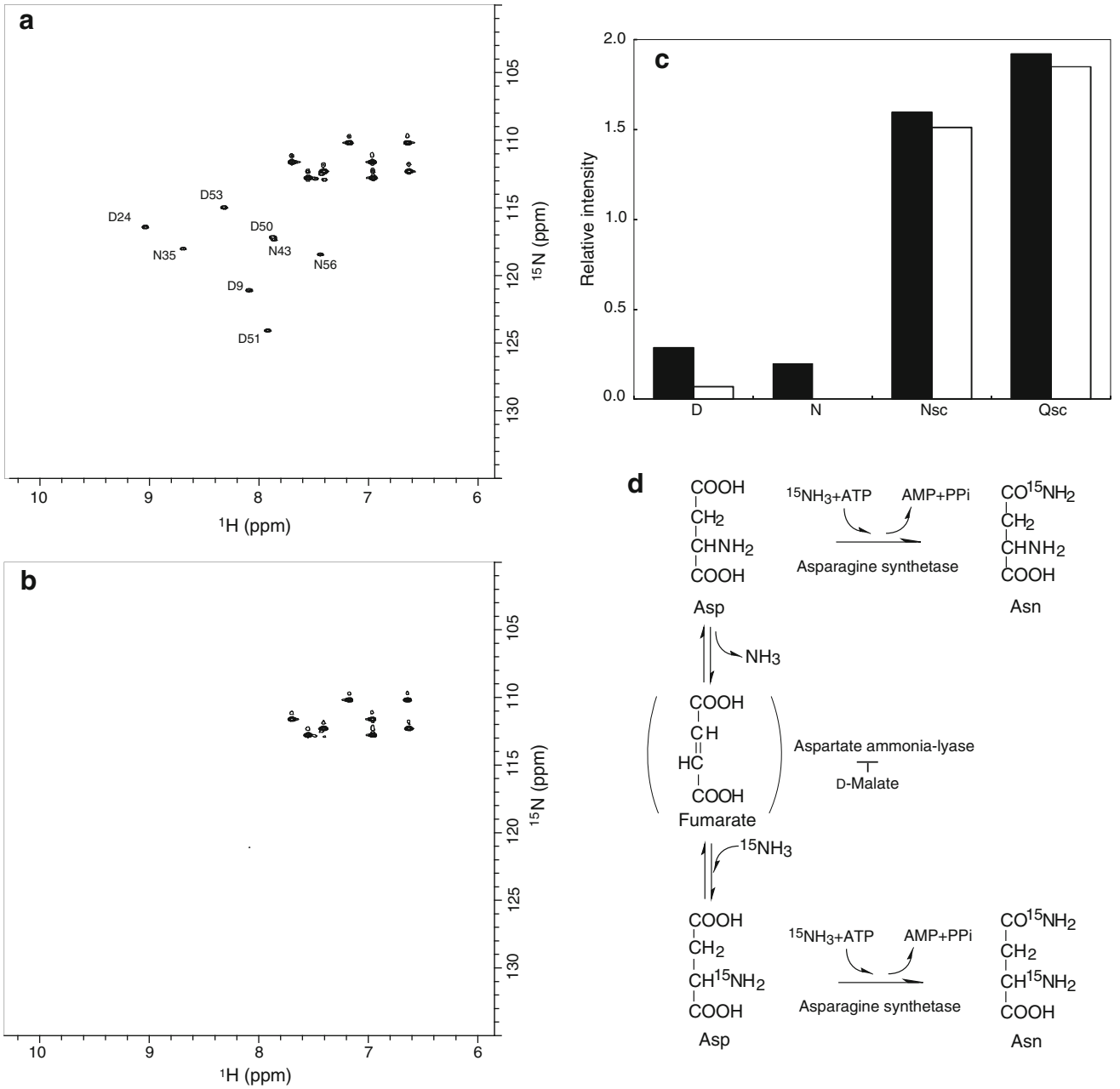

Fig. 3 Side-chain amide selective ^{15}N-labeled UBA protein. ^{1}H-^{15}N HSQC spectra (**a**, **b**) and comparison of cross peak intensities for the main-chains of Asp and Asn, and the side-chains of Asn and Gln residues (**c**) of UBA proteins prepared without (**a**, *filled bars* in **c**) and with (**b**, *open bars* in **c**) D-malate, respectively. The cross peak intensities are normalized to the average intensity. Metabolic pathways involved in the isotopic scrambling of the main-chain and side-chain amides of Asp and Asn (**d**)

Trp, and Cys can be synthesized from their precursors by endogenous metabolism in the CF reaction solution.

Finally, using the optimized conditions with 3 mg/mL of ^{15}N-labeled AAM, 0.3 mM ^{15}N-indole, 1 mM Na_2S, 0.2 mM PLP, 0.1 mM AcCoA, and 27 mM ^{15}N-labeled ammonium acetate, the productivity for both CAT and GFP became almost equal to that under standard conditions (Fig. 2c, d).

Side-chain amide selective labeling of Asn and Gln residues

Asn and Gln residues play important structural roles in protein–protein and protein-substrate interactions, because their side-chain amide groups can act as both hydrogen bond acceptors and donors on the surface of proteins or in the active site of enzymes. For example, observation

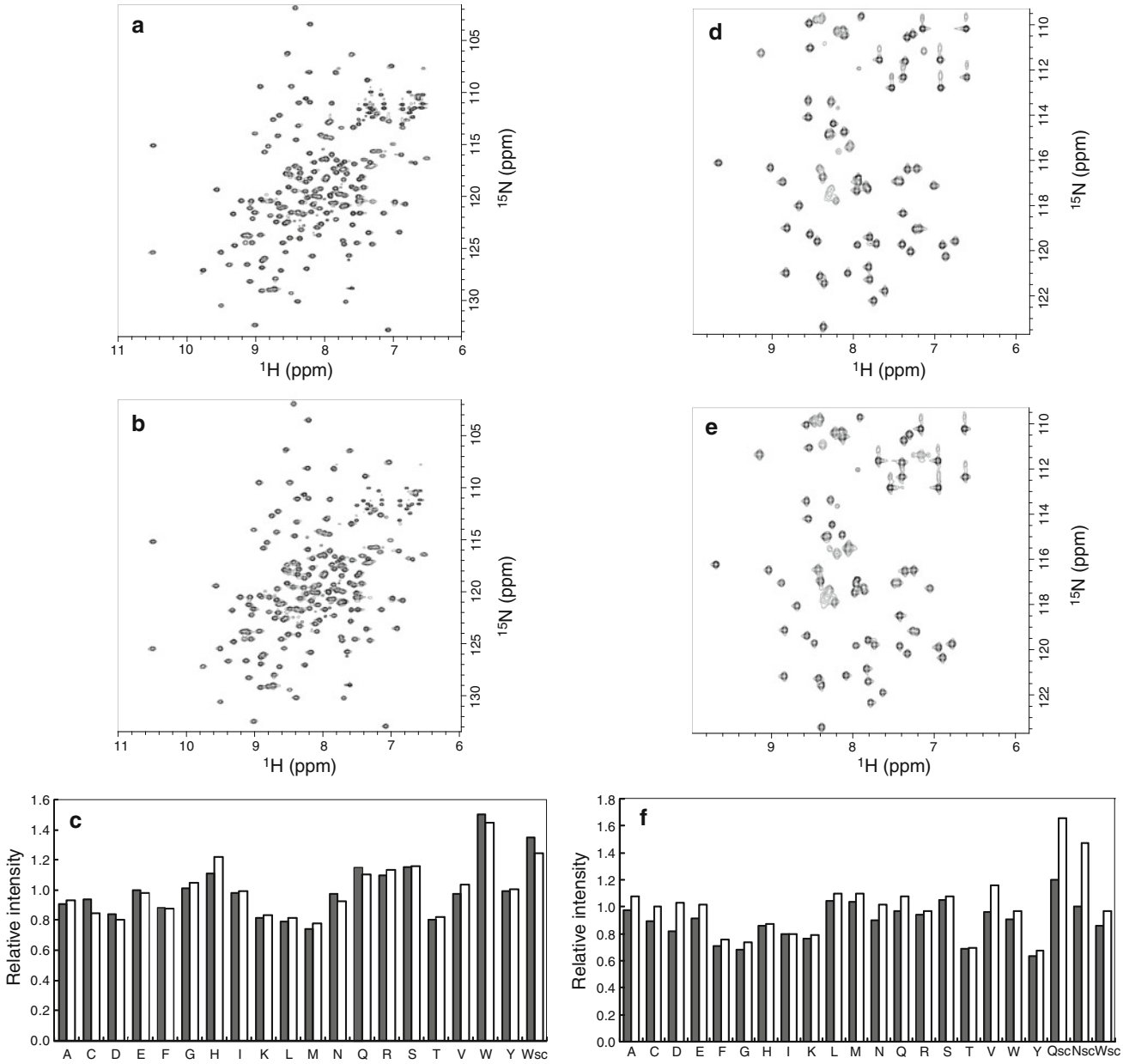

Fig. 4 ^{1}H-^{15}N HSQC spectra (**a**, **b**, **d**, **e**) and comparison of cross peak intensities in the spectra (**c**, **f**) of ^{15}N-labeled Ras(Y32 W) (**a**–**c**) and UBA (**d**-**f**) proteins synthesized with 20 kinds of ^{15}N-labeled amino acids (**a**, **d**, *filled bars* of **c**, **f**) and ^{15}N-algal AAM, ^{15}N-indole, Na_2S, and ^{15}N- ammonium acetate (**b**, **e**, *open bars* of **c**, **f**), respectively. The averaged cross peak intensities of each amino acid residues are normalized to the average intensity of all cross peaks. Data for overlapping cross peaks are excluded from the average intensity (**c**, **f**)

of the side-chain amide groups of Asn and Gln residues was useful to understand the interaction of hen egg-white lysozyme with its substrate (Higman et al. 2004).

As expected from biosynthetic pathways of Asn and Gln in *E. coli* (Fig. 1), Asn and Gln were successfully synthesized from Asp and Glu during the CF reaction, respectively (Fig. 2a, lane 1, 3). Likewise, we expected that the selective ^{15}N-labeling of the side-chain amide groups of Asn and Gln residues was possible by utilizing endogenous metabolic conversion in the CF reaction from Asp, Glu, and ^{15}N-labeled ammonium ion. However, in addition to 4 pairs of cross peaks of the side-chain amide groups, 3 from Asn residues and 1 from Gln residue, those of main-chain amide groups of Asp and Asn residues were also observed in the ^{1}H-^{15}N-HSQC spectrum (Fig. 3a, c). This indicated that both the main-chain and side-chain amide groups of Asp and Asn residues exchanged directly and/or indirectly with the amino group of the ^{15}N-labeled ammonium ion during the CF reaction. This phenomenon could be explained by the reaction catalyzed by L-aspartate ammonia-lyase (AAL)

(EC 4.3.1.1), which reversibly deaminates Asp to yield fumarate and ammonia (Fig. 3 d). The addition of D-malate, as an inhibitor of AAL, to the CF reaction solution successfully prevented this isotopic scrambling (Fig. 3b–d). The side-chain selective labeling of Asn and Gln residues using the cell-based expression method was previously reported (Tate et al. 1992); however, severe isotopic scrambling among Asp, Asn, Glu, and Gln residues was also reported for the cell-based method (Takeuchi et al. 2007; Tong et al. 2008). In this study, we have successfully developed an inexpensive method for the highly selective side-chain ^{15}N-labeling of Asn and Gln residues, by using the CF in which the amino acid metabolism causing isotopic dilution and scrambling in both cell-based and conventional CF-based expression was regulated by its inhibitor.

Uniformly ^{15}N-labeled proteins synthesized with the optimized conditions and NMR measurements

^{15}N-labeled Ras(Y32 W) protein and UBA protein were prepared using the large-scale dialysis mode of the CF reactions, with the optimized conditions developed in this study (Ras(Y32 W)/16AAM and UBA/16AAM) and the standard conditions (Ras(Y32 W)/20AAM and UBA/20AAM), respectively. In the ^{1}H-^{15}N HSQC spectra, the cross peak intensities of Ras(Y32 W)/16AAM and UBA/16AAM were almost the same as those of Ras(Y32 W)/20AAM and UBA/20AAM, respectively (Fig. 4a–f). These results demonstrated that the four expensive amino acids, Gln, Asn, Trp, and Cys, could be efficiently synthesized from other sources, such as amino acids and precursors, by the endogenous metabolic enzymes in the CF reaction solution.

The calculated cost of the related compounds for SI labeling of proteins using the CF, according to the reagent prices listed in the web catalogues of CIL (http://www.isotope.com/cil/) and Sigma–Aldrich (http://www.sigmaaldrich.com/chemistry/stable-isotopes-isotec.html), revealed that our newly developed labeling method with the reduced set of amino acids saved nearly 40% of the cost of the uniformly ^{13}C, ^{15}N-labeled protein production (Fig 5). Our method will be more effective in ^{2}H, ^{13}C, ^{15}N-labeling of proteins, because the SI-labeled compounds certainly cost more than the ^{15}N-labeled and ^{13}C, ^{15}N-labeled compounds.

Over the past few years, various improvements and optimizations have been made for the SI-labeling of proteins using the CF. The *E. coli* S30 extract (Spirin et al.

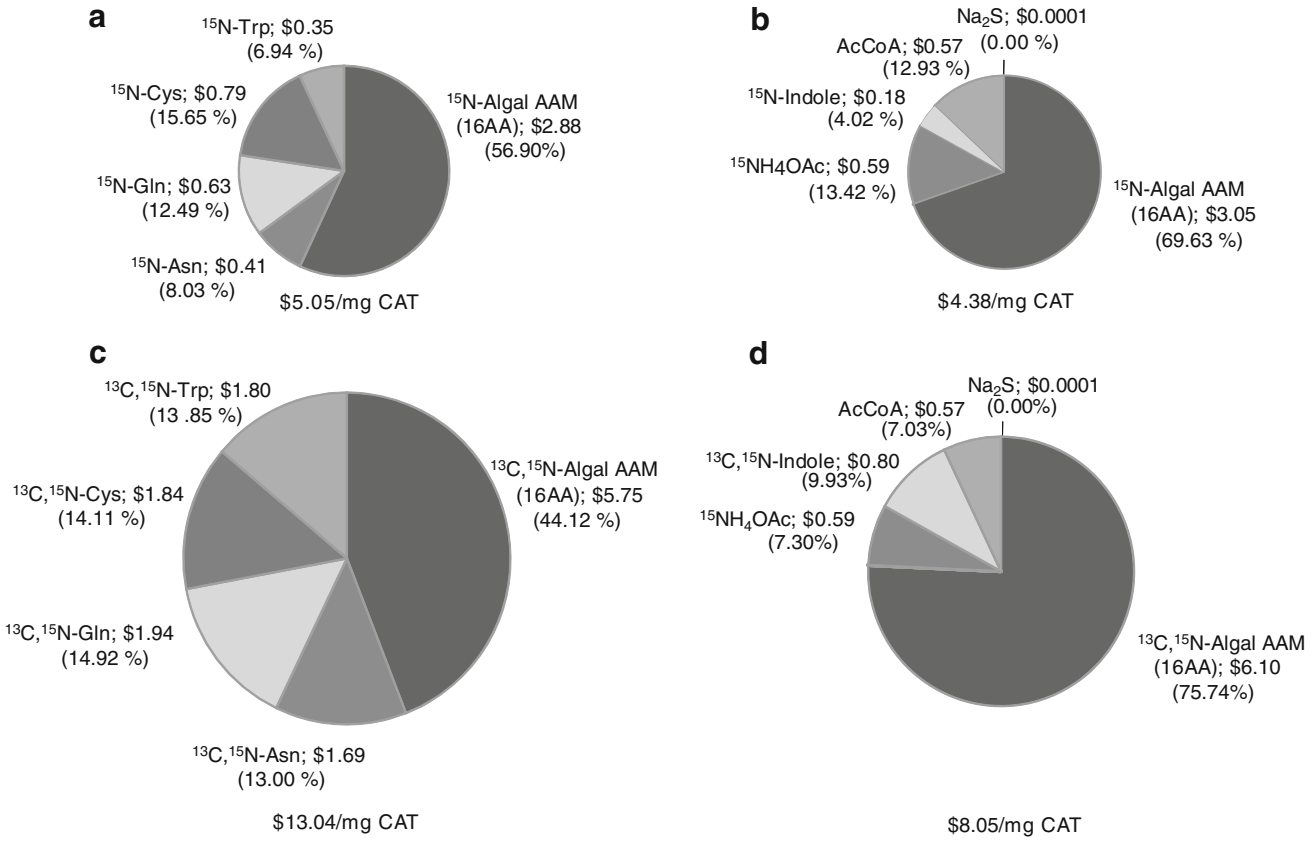

Fig. 5 Cost of stable-isotope related compounds required for the production of 1 mg of ^{15}N-labeled (**a**, **b**) and ^{13}C, ^{15}N-labeled (**c**, **d**) CAT proteins, using the CF reaction with the standard (**a**, **c**) and optimized (**b**, **d**) conditions, respectively

1988) for the CF contains not only essential substances for translation but also various housekeeping enzymes, such as nucleases (Seki et al. 2009; Yang et al. 1980), proteases, and amino acid transaminases (Ozawa et al. 2004). The major enzymes involved in amino acid degradation in the CF extract were identified, and their deficient mutant strains were created for the CF (Calhoun and Swartz 2006). Some inhibitors to suppress the metabolic pathways related to the isotope dilution were also investigated (Morita et al. 2004; Ozawa et al. 2004). These studies indicated that a thorough understanding of amino acid metabolism in the CF was especially important for establishing the CF with low amino acid consumption. Therefore, a technique to monitor during the CF reaction, using a UPLC amino acids analysis system with AccQ-Tag, was established in this study, which greatly contributed to clarifying the metabolic amino acid degradation and conversion in the CF. In almost all of the previous studies on cost reduction of SI labeling, amino acid metabolism was inhibited and/or removed, in order to suppress the consumption of expensive amino acids. On the other hand, in the present study, amino acid metabolism was utilized to generate expensive amino acids from inexpensive sources during the CF reaction. Our approach successfully achieved both convenient and cost-effective SI-labeling of proteins using the CF. The combination of suppressing undesirable metabolic enzymes by the chemical inhibitor and/or the genetic engineering and activation of useful enzymes for metabolic conversion/generation presented in this study will enable more efficient consumption of SI-labeled compounds, and therefore, better cost-effective SI-labeling of proteins with the CF. This will certainly offer more opportunities to produce SI-labeled proteins for NMR analysis using the CF.

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