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ARTICLE

Suppression of phospholipid biosynthesis by cerulenin in the condensed Single-Protein-Production (cSPP) system

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Abstract Using the single-protein-production (SPP) system, a protein of interest can be exclusively produced in high yield from its ACA-less gene in *Escherichia coli* expressing MazF, an ACA-specific mRNA interferase. It is thus feasible to study a membrane protein by solid-state NMR (SSNMR) directly in natural membrane fractions. In developing isotope-enrichment methods, we observed that ^{13}C was also incorporated into phospholipids, generating spurious signals in SSNMR spectra. Notable, with the SPP system a protein can be produced in total absence of cell growth caused by antibiotics. Here, we demonstrate that cerulenin, an inhibitor of phospholipid biosynthesis, can suppress isotope incorporation in the lipids without affecting membrane protein yield in the SPP system. SSNMR analysis of ATP synthase subunit *c*, an *E. coli* inner membrane protein, produced by the SPP method using cerulenin revealed that ^{13}C resonance signals from phospholipid were markedly reduced, while signals for the isotope-enriched protein were clearly present.

Keywords cSPP · Membrane protein · Phospholipid biosynthesis · Cerulenin · SSNMR

Introduction

Using the single-protein-production (SPP) system that exploits MazF, an mRNA interferase which cleaves mRNA at the ACA sequences, a protein of interest, coded by an mRNA without use of the ACA triplets, can be selectively produced in *E. coli* cells while the production of other cellular proteins is almost completely suppressed(Suzuki et al. 2005). Addition of ^{13}C-glucose in the medium at the time of expression can therefore allow selective isotopic enrichment of the protein of interest with ^{13}C without incorporation in any other cellular proteins(Mao et al. 2010). The presence of a specifically isotopically-enriched protein in the context of an intact cell provides exciting opportunities in biophysical studies, particularly for NMR experiments.

Whole cell NMR has been used for decades (Hayashi et al. 1981). Considering that NMR spectral information may sometimes be more biologically relevant for proteins studied inside of the cell, the field was somewhat revived through the work of the Dotsch group (Serber et al. 2001; Serber et al. 2004; Serber et al. 2006). Interesting recent elaborations have allowed it to be used to probe protein–protein interactions inside of the cell (Burz et al. 2006). These methods, however, are not without complications. A few years ago, an attempt was made to determine the

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dynamic behavior of chymotrypsin inhibitor 2 (CI2) and apocytochrome-b5 in living *E. coli* (Bryant et al. 2005, 2006). A later experiment showed that most of the signals that had been observed arose from protein that had leaked out of the cells and into the surrounding medium (Pielak 2007). This might or might not be general phenomenon, and other complexities can arise. In one study, *E. coli* cells that were producing CI2 or αSN in alginate microcapsulesm (which prevented protein leakage) showed normal spectra of αSN, but no detectable CI2 spectra (Li et al. 2008). Possibly the viscosity of the medium or complexation of the proteins caused this phenomenon. Systematic relaxation studies have been carried out hopefully will help to clarify this puzzle (Li et al. 2008). If many proteins are indeed immobilized *in vivo*, solid-state NMR (SSNMR) methods could be very useful, and allow one to bypass some of the complexities encountered in these studies.

^{13}C-enrichement using ^{13}C-glucose as a precursor results in ^{13}C-incorporation into other macromolecules besides proteins, leading to additional NMR signals in whole cell or membrane-extract preparations that might be unwanted. Of specific interest to membrane protein studies is the fact that ^{13}C is incorporated into phospholipids with a typical turnover of 3–5% per generation in *E. coli* cells during logarithmic growth(Rock 1984). This process also occurs in the SPP system. Such ^{13}C-enriched lipids generate spurious signals in NMR spectra of membrane-containing cellular fractions produced by the SPP system. In particular, strong signals from phospholipid observed in many kinds of ^{13}C-detected multidimensional spectra, can overlap with spectral regions of interest, and are problematic for detection of weaker peaks in the spectra and for data processing. These signals have presented a major obstacle in the structural studies of membrane proteins by ^{13}C-detected SSNMR experiments using natural membrane fractions obtained from the SPP system.

The antibiotic cerulenin is known to inhibit phospholipid biosynthesis by blocking FabB and/or FabF in the elongation step of fatty acid biosynthesis (Heath et al. 2001). In this paper we assessed whether cerulenin effectively inhibits the biosynthesis of phospholipid in the SPP system. Furthermore we address the issue of whether suppressing lipid biosynthesis at the time of production of an intrinsic membrane protein will adversely affect the product of the protein of interest, focusing on the protein ATP synthase subunit *c*, an *E. coli* inner membrane protein. The results demonstrate high level production of selectively ^{13}C-enriched ATP synthase subunit *c* in natural membrane fractions in the presence of cerulenin antibiotic, without ^{13}C-enrichment of membrane phospholipids.

Materials and methods

Protein expression in the condensed SPP (cSPP) system

E. coli BL21 (DE3) transformed with pACYC*mazF* (Suzuki et al. 2005) and pColdI(SP-4) (Suzuki et al. 2007) harboring the target gene was grown in M9-glucose medium at 37°C (Suzuki et al. 2007). When the culture's OD_{600} reached 0.5–0.6, the culture was chilled on ice for 5 min and then moved to 15°C for 45 min for cold-shock acclimation. To condense the culture, cold-shock treated cells from a 1-l culture were harvested by centrifugation at 3000×g for 30 min at 4°C. The cell pellet was then gently suspended in 50 ml of M9-glucose medium (20-fold condensation), containing 1 mM IPTG. The cells were incubated overnight at 15°C to induce the target protein with shaking.

Preparation of uniformly ^{15}N, ^{13}C-enriched ATP synthase subunit *c* (AtpE)

After cold-shock treatment, the expression of both MazF from pACYC*mazF* and subunit *c* from pColdI(SP-4)(Suzuki et al. 2007) harboring the gene for subunit *c* were induced with 1 mM IPTG in M9 medium for 3 h. The cells were then harvested by centrifugation at 3000 × g for 30 min at 4°C(Suzuki et al. 2007). The cell pellet was then washed with 50 ml of M9 phosphate buffer and suspended in 50 ml of M9 minimal medium (20-fold condensation) containing 1 g/l $^{15}NH_4Cl$, 4 g/l uniformly ^{13}C-enriched glucose (Cambridge Isotope Laboratories, Inc.). Induction of ATP synthase subunit *c* was continued at 15°C with shaking for overnight. The cells from the cultures were harvested by centrifugation and the cell pellets were stored at −80°C.

^{14}C-acetic acid incorporation and phospholipid extraction

Five μci of ^{14}C-acetic acid (58 μci/μmol, from PerkinElmer) was added to 1 ml cell culture at each time point and the pulse labeling was carried out for 20 min. The cell culture was transferred to a micro tube and centrifuged at 12,000×g, for 30 s at 4°C. The cell pellet was suspended in 500 μl of 100 mM potassium phosphate buffer by vortex. 250 μl of methanol was added to the cell suspension, which was then kept at −20°C for further phospholipid extraction. Phospholipid were extracted by the method of Kanfer and Kennedy with modification (Kanfer and Kennedy 1963). Two hundred microliters of the methanol-cell suspension was transferred to a 10-ml tube to which 266 μl of methanol was further added. To this mixture, 180 μl of chloroform

was added and phospholipids were then extracted by a vortex for 3 min. After keeping the tube at room temperature for 10 min, another 180 µl of chloroform and 180 µl of 0.1 M potassium phosphate buffer (pH 7.2) were added. The mixture was further extracted by a vortex for 1 min and then centrifuged at 2,000 rpm (Sorvall RC-5B) for 10 min. The upper phase (about 300 µl) was taken, to which 266 µl of chloroform: methanol: 0.1 M potassium phosphate buffer (3:48:47) were added. The mixture was extracted with a vortex for 3 min and then centrifuged at 2,000 rpm (Sorvall RC-5B) for 10 min. The upper phase was removed with a pipet. The lower chloroform phase contains extracted phospholipid. 200 µl of this lower chloroform phase was transferred to a scintillator tube. After evaporating chloroform, scintillation fluid was added to determine the ^{14}C radioactivity for each sample with a LKB WALLAC 1209 RackBeta liquid scinitillation counter.

Membrane preparation

The cell pellet from a 1-l culture was suspended in 10 ml of 50 mM Tris buffer (pH 7.4). The cells were lysed by a French press at 15,000 psi, followed by centrifugation at 5, 000 rpm, 15 min at 4°C (Sorvall RC-5B refrigerated superspeed centrifuge) to remove cell debris and inclusion bodies. The membrane fraction was then collected by centrifugation at 100,000 × g for 60 min at 4°C. The membrane pellet was suspended in 1 ml of 50 mM Tris buffer (pH 7.4) by sonication. Fifty µl of the membrane suspension was used for further separation of the inner and outer membranes. The remaining suspension was divided into two equal 500-µl fractions. These fractions were centrifuged at 100,000 × g for 60 min at 4°C and the membrane pellets were kept at −80°C.

Separation of inner and outer membrane fractions

In order to determine the localization of a target membrane protein, the inner membrane fraction is separated from the outer membrane fraction by Sodium-Lauryl Sarcosinate (Sarkosyl) (Filip et al. 1973). Two hundred micro liter of 50 mM Tris buffer (pH 7.4) was added to the 50-µl membrane suspension. Subsequently, 250 µl of 1% Sarkosyl was added and the mixture was incubated at room temperature for 20 min to dissolve the inner membrane proteins. The inner membrane fraction was then separated as the supernatant from the pellet of the outer membrane fraction by centrifugation at 135,000 × g for 30 min at 4°C.

NMR spectral measurements

Uniformly ^{13}C, ^{15}N-enriched subunit *c* samples in the *E. coli* membrane prepared as described above were equilibrated at 98% humidity and packed into 4 mm Varian SSNMR rotors. Measurements were carried out on a Varian Infinity Plus 400 MHz spectrometer with a 4-mm SSNMR HX probe. The experiments were conducted at ^{1}H resonance frequency of 396.78 MHz and ^{13}C resonance frequency of 99.78 MHz, with magic angle spinning frequency of 9 kHz and apparent temperature of −40°C. Cross polarization (CP) steps were carried out with 50 kHz field strength on ^{13}C channel and 59 kHz on ^{1}H channel. ^{1}H TPPM decoupling (Bennett et al. 1995) field of 100 kHz was applied during the detection period of 15 ms with spectrum width of 30.03 kHz. The 1D ^{13}C spectra were signal averaged for 10 h for subunit *c* with cerulenin sample and 3.5 h for subunit *c* without cerulenin sample.

Results

Cell growth inhibition by cerulenin

Several antibiotics are known to be inhibitors of certain steps in the fatty acid synthesis pathway in *E. coli* (Heath et al. 2001). Among the antibiotics tested in this study, we found that cerulenin was most effective to show clear inhibition on cell growth of *E. coli* (data not shown). Cerulenin has been known to inhibit cell growth of *E. coli* by blocking unsaturated fatty acid biosynthesis (Buttke and Ingram 1978). We examined the effect of different concentrations of cerulenin on cell growth at two different temperatures, 15°C and 37°C as shown in Fig. 1. YnaJ, an *E. coli* inner membrane protein, was chosen as a target protein since it is expressed well in the SPP system (Mao et al. 2009). All the ACA sequences in this gene were altered to non-MazF cleavable sequences without changing the amino acid sequence of YnaJ according to the method described previously (Suzuki et al. 2007). *E. coli* BL21 (DE3) transformed with pACYC*mazF*(Suzuki et al. 2005) and pColdI(SP-4)(Suzuki et al. 2007) harboring YnaJ was grown in M9-glucose medium at 37°C until OD_{600} reached 0.5–0.6. The culture was then shifted to 15°C for 45 min in order to acclimate the cells to cold temperature. Then the cold-shocked culture was spotted onto M9-glucose plates which contains different concentrations of cerulenin in each well from 0 to 100 µg/ml (Fig. 1), followed by incubation at 15°C for 3 days or 37°C overnight, respectively. The results showed that at 15°C cells can survive at 10 µg/ml of cerulenin, but not at 25 µg/ml or higher. However, at 37°C, cell growth was inhibited by cerulenin at 50 µg/ml or higher concentrations. Since in the SPP system, a target gene was cloned in pCold(SP-4) vectors(Suzuki et al. 2007), expression of the target protein is induced at 15°C. Therefore, 25 µg/ml of cerulenin, sufficient to arrest normal *E. coli* bacterial growth, was chosen

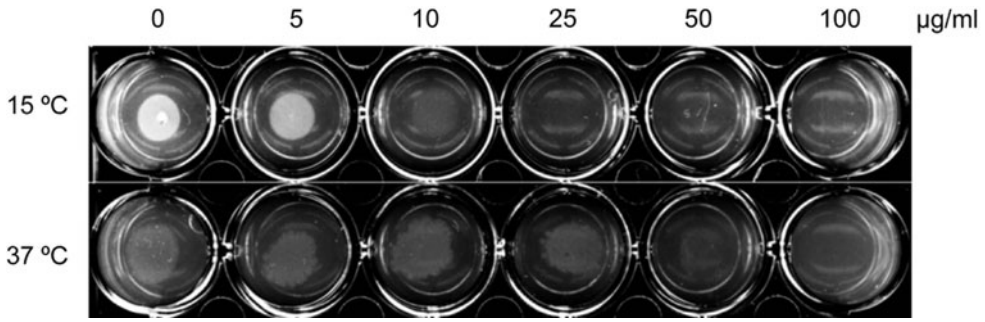


Fig. 1 Cell growth blocked by cerulenin. The cold-shock culture of *E. coli* BL21 (DE3) transformed with pACYC*mazF* (Suzuki et al. 2005) and pColdI(SP-4) (Suzuki et al. 2007) harboring YnaJ was spotted on M9-glucose plates. Each well contains cerulenin from 0 to 100 μg/ml as indicated. One plate was incubated at 15°C for 3 days and the other was incubated at 37°C overnight, corresponding to the upper and lower *panel*, respectively

to further test its inhibitory effect on phospholipid biosynthesis and protein production by the SPP system at 15°C.

Phospholipid biosynthesis is significantly inhibited by 25 μg/ml of cerulenin in the SPP system without affecting protein yield

In the SPP system, cell growth is completely arrested. However, these quasi-dormant cells are still capable of producing a target protein and do so in a selective and efficient fashion (Suzuki et al. 2005). As previously described(Suzuki et al. 2007), after the culture's OD_{600} reaches 0.5–0.6, the cell culture was shifted to 15°C and incubated for 45 min to allow the cells to acclimate to the low temperature. In this case, however, the cold-shocked culture was split into three flasks with 25 μg/ml cerulenin, 1 mM IPTG, and 25 μg/ml cerulenin plus 1 mM IPTG, respectively. One ml of culture was taken from each flask and subjected to pulse labeling of ^{14}C-acetic acid after 2 h and overnight incubation at 15°C. One ml of the original culture was taken as a "0-h time point". After extracting the phospholipid, the radioactivity of each sample was counted and plotted in Fig. 2a. After 2 h incubation, the level of phospholipid production was reduced to 49% with cerulenin only and 17% with both of cerulenin and IPTG (compared to the zero time point). On the other hand, the production of phospholipid was elevated by 43% with addition of IPTG alone, i.e. the condition of the regular SPP system (Fig. 2a). A higher degree of inhibition of phospholipid synthesis due to cerulenin can be observed after an overnight incubation. The level of phospholipid production was reduced to 7% with addition of cerulenin and 4% with addition of both cerulenin and IPTG. On the other hand, for cultures with only IPTG but no cerulenin, the lipid synthesis activity was maintained near the same level as observed at the 2 h timepoint. Overall, we achieved around 95% inhibition of phospholipid biosynthesis with addition of 25 μg/ml cerulenin to the SPP system.

We next addressed whether the protein yield of the SPP system is affected in the presence of cerulenin. 1-ml cultures were taken from the original cold-shocked culture and cultures of the three different conditions described above. The cells were collected and subjected to SDS–PAGE, followed by Coomassie Brilliant Blue staining. Without IPTG (as indicated -/- and -/cerulenin for lanes 1 and 2, Fig. 2b, respectively), there is no YnaJ produced. Addition of only IPTG served as a positive control shows very good expression of YnaJ (Fig. 2b, lane 3). In the presence of both cerulenin and IPTG, we observed the same expression level of YnaJ as in the absence of cerulenin (Fig. 2b, cf. lanes 3 and 4). Thus, at cerulenin concentrations (25 μg/ml) sufficient to inhibit both cell growth and ^{14}C incorporation into phospholipids, target protein production by the SPP system is not reduced. This constitutes a novel and significant improvement in the SPP system, and indicates the great potential of the SPP system to be applied to SSNMR studies.

NMR spectra indicate effective inhibition of phospholipid biosynthesis with 25 μg/ml cerulenin in the SPP system

One of the unique features of the SPP system is that the cell culture can be condensed without reducing protein yield, allowing for significant reduction in the cost of isotope labeling(Suzuki et al. 2006). pColdI(SP-4)*atpE* was transformed into *E. coli* BL21(DE3) which contained pACYC*mazF* harboring a *lac*-inducible *mazF* gene(Suzuki et al. 2005). A 1-l exponentially growing culture at 37°C (OD_{600} of 0.5) was cold-shocked at 15°C for 1 h, then 1 mM IPTG was added to induce the mRNA interferase, MazF. The culture was further incubated at 15°C for 3 h, followed by 20-fold condensation in M9 minimal medium (to 50 ml) containing $^{15}NH_4Cl$, ^{13}C-glucose, 1 mM IPTG, and 25 μg/ml cerulenin (cSPP system). The condensed

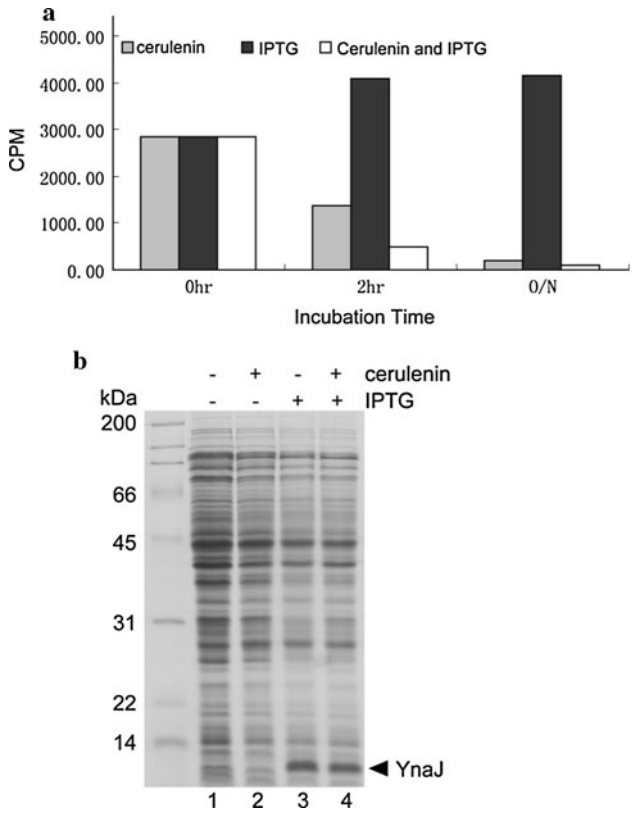


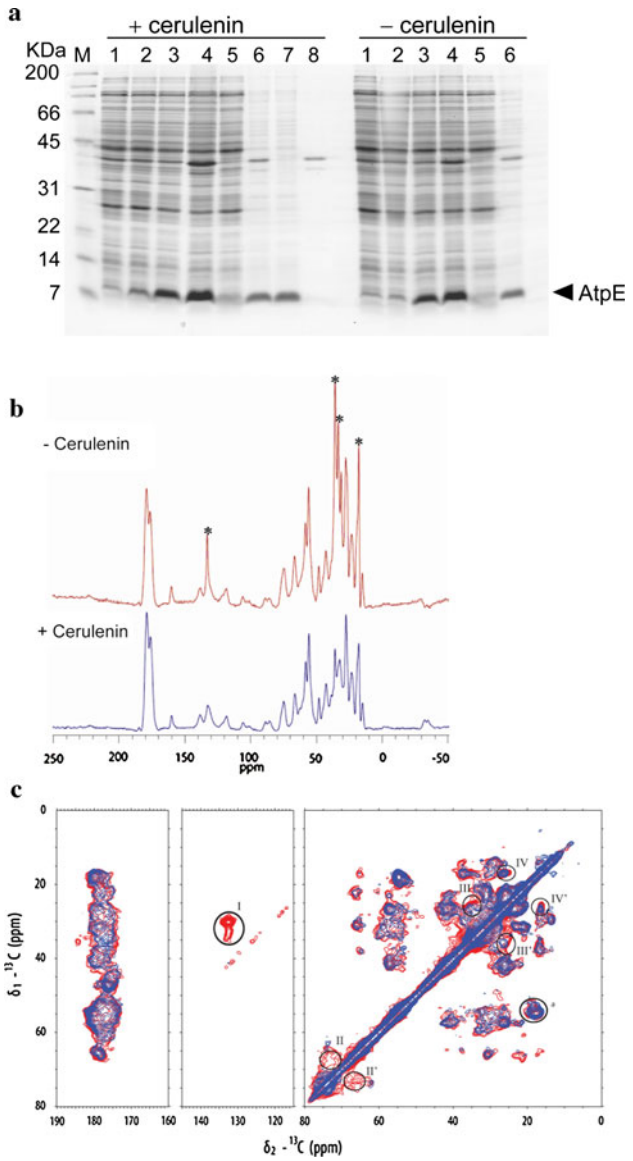
Fig. 2 Effect of cerulenin in phospholipid biosynthesis and protein production in the SPP system. Phospholipid biosynthesis was blocked by 25 μg/ml cerulenin without affecting protein yield in the SPP system. The cold-shocked culture, as described in Fig. 1, was divided into three flasks which contain 25 μg/ml cerulenin, 1 mM IPTG, and 25 μg/ml cerulenin plus 1 mM IPTG, respectively. The culture taken from the original flask is −/− control. A 1 ml culture from each flask at different time points (0, 2 h and overnight) was added to tubes that contained 5 μci of ^{14}C-acetic acid (58 μCi/μmol). The tube was incubated at 15°C for 20 min with shaking. The cells were collected by centrifugation at 12,000×g, 4°C, for 30 s. The cell pellet was then suspended in 500 μl of 0.1 M potassium phosphate buffer (pH 7.2). 250 μl of methanol was added. The mixture was kept at −20°C, followed by phospholipid extraction. The radioactivity was counted to measure the synthesis of phospholipid. **a** *Gray*, 25 μg/ml cerulenin; *Black*, 1 mM IPTG and *White*; 25 μg/ml cerulenin plus 1 mM IPTG. **b** Cells from a 1-ml culture were collected for SDS–PAGE. Control of −/− was from the original cold-shocked culture. All the other samples were from the overnight culture under the three different conditions. Position of YnaJ is indicated by an *arrowhead*

culture was incubated at 15°C for about 24 additional hrs to produce uniformly ^{15}N and ^{13}C-enriched subunit *c*, which is the *c* subunit of *E. coli* ATP synthase F_0. The total membrane fraction was prepared from the condensed culture. Their SDS PAGE analyses are shown in Fig. 3a. Paralleled expression of subunit *c* in the SPP system with or without cerulenin indicated similar expression levels for the two conditions (Fig. 3a). Subunit *c* was expressed well in the cSPP system comparing lane 3, induced, to lane 1, −3 h. The protein remained in the supernatant fraction after low speed centrifugation (lane 4), indicating that subunit *c* was not produced as inclusion bodies. This supernatant fraction was then centrifuged at 100,000 × g, 4°C for 1 h to separate the cytoplasmic soluble fraction (lane 5) and the membrane fraction (lane 6). Subunit *c* was found to be exclusively localized in the membrane fraction and it appears to be the most abundant protein in the inner membrane on the basis of Coomassie Brilliant Blue staining (lane 7). In order to increase the relative concentration of subunit c in the membrane pellet, the membrane fraction was treated with 10 mM EDTA and 80 μg/ml lysozyme to remove the cell wall, which resulted in reduction of the pellet weight by approximately 25% without losing any protein (data not shown).

SSNMR ^{13}C cross polarization 1D spectra were then collected from the membrane pellet containing uniformly ^{15}N and ^{13}C-labeled subunit *c* prepared with or without cerulenin by the SPP system. Signals from isotopically enriched phospholipid can be identified on the 1D spectrum of the sample prepared without cerulenin. There are four peaks listed out by "*" arising from lipid carbon tails (Fig. 3b). By comparing the upper spectrum in red (minus cerulenin) with the lower spectrum in blue (plus cerulenin), we observe significant decrease of intensity for those four ^{13}C resonance peaks due to the phospholipids. These signals would interfere with further studies involving certain amino acid types; for example, signal coming from the unsaturated tails of lipids overlap with resonances of Phe or Tyr; signals coming from the glycerol head group of lipids obscure the signals from Thr; signals coming from the saturated bonds of lipid tails obscure the signals from Ile and Leu. These unwanted signals from phospholipid were eliminated from the spectra by the addition of cerulenin during protein expression, as illustrated in Fig. 3b. This result demonstrates the very efficient inhibition of phospholipid biosynthesis by cerulenin. It is further analyzed by recording 2D ^{13}C-^{13}C spectra from the same samples. In Fig. 3c, the cross peaks from isotopically enriched lipids are indentified, which are unsaturated tails (I), glycerol head group (II and II′) and saturated tails (III, III′, IV and IV′). The lipid signal in the "+cerulenin" sample (blue) was significantly smaller than the "–cerulenin" sample (red), with relative intensity decreased to be 54% (peak IV′) in lipid saturated tails and 2% (peak I) in lipid unsaturated tails. The relative intensity of lipid signals was determined as ratio of lipid signal to protein signal (peak a, Ala Cβ-Cα cross peak, Fig. 3c).

Discussion

In our initial studies, we found that although the target protein was the only protein labeled in the SPP system, the



◀ **Fig. 3** Uniformly ^{13}C, ^{15}N-labeling of ATP synthase subunit *c* (AtpE) in the SPP system with or without cerulenin. **a** Uniformly ^{13}C, ^{15}N-labeled subunit c was produced and by the SPP system as described above in Fig. 2. M, molecular weight markers; *lane 1*, −3 h, add 1 mM IPTG to start induction of MazF and subunit c; *lane 2*, 0 h, exchange to the M9 medium containing ^{13}C-glucose and $^{15}NH_4Cl$ (with or without 25 μg/ml cerulenin); *lane 3*, whole cell after overnight induction; *lane 4*, cell lysate after removing unbroken cells and inclusion bodies; *lane 5*, soluble fraction; *lane 6*, membrane fraction; *lane 7*, inner membrane fraction; and *lane 8*, outer membrane fraction. The position of subunit c is indicated by an *arrowhead*. **b** 1D ^{13}C cross polarization spectra of *E. coli* ATP synthase subunit *c* prepared by the SPP system with (*blue*) and without (*red*) cerulenin. The peaks marked with *asterisks* are signals from lipids. Both spectra were recorded on a Varian Infinity Plus 400 MHz spectrometer, at the magic angle spinning speed of 9 kHz and apparent temperature of −40°C. The free induction decays were recorded for 15 min with the proton decoupling field of 100 kHz. The times of signal averaging were 10 h for the sample prepared with cerulenin and 3.5 h for the sample prepared without cerulenin. The ^{13}C NMR signals from phospholipids are much smaller in the spectrum of the sample produced with cerulenin antibiotic. **c** 2D ^{13}C–^{13}C spectra of *E. coli* ATP synthase subunit *c* prepared by Single-protein Production method with (*blue*) and without (*red*) cerulenin. The cross peaks from isotopically enriched lipids can be indentified: unsaturated tails (I), glycerol head group (II and II′) and saturated tails (III, III′, IV and IV′). Both spectra were signal averaged for 38 h on a Varian Infinity Plus 400 MHz spectrometer, at the magic angle spinning speed of 9 kHz and apparent temperature of −40°C. The spectra were acquired for 15 min in the direct dimension and 11.5 min in the indirect dimension

isotopes were also incorporated into the lipid component of *E. coli.* It had been reported that cerulenin, and also some other antibiotics such as thiolacomycin and Isoniazid(Heath et al. 2001), can inhibit certain steps of the lipid biosynthesis pathway in *E. coli* (Heath et al. 2001). After screening the different antibiotics of various concentrations in the SPP system, we found 25 μg/ml of cerulenin can inhibit phospholipid biosynthesis very efficiently in the SPP system, which is further demonstrated by comparing 1D ^{13}C-detected SSNMR spectra. On the other hand, protein yield in the condensed SPP (cSPP) system is not affected by addition of cerulenin. In the SPP system, cell growth is completely arrested, causing the quasi-dormancy state (Suzuki et al. 2005). These arrested cells become bioreactors, capable of producing only the protein of interest. Antibiotics like cerulenin targeting at lipid biosynthesis can lead to cell death in normal *E. coli,* because no more lipids are available for cell growing. Apparently, however, this phenomenon does not occur in the SPP system. This unique observation of the SPP system provides an opportunity to study the antibiotic physiology of cerulenin under conditions that are usually lethal to *E. coli*. It can also be expanded to other antibiotics that block certain pathways in *E. coli* but not affect cell viability in the SPP system.

Our improved SPP system has the following unique features over the conventional method of production of membrane proteins. First of all, only a protein of interest is uniformly labeled and assembled into native membrane of *E. coli*. We can use the membrane fraction directly for data collection and may determine the structure of membrane proteins in a minimally altered form. Secondly, it has been demonstrated that incorporation of isotopes and toxic amino acid analogues, like selenomethione and fluorophenylalanine, are very efficient(Suzuki et al. 2006). Thirdly, the cell culture can be condensed 20- to 50-fold in the SPP system without reducing protein yields, the cost saving by which is more than 95% (Suzuki et al. 2007). With this improvement, we may be able to explore a new avenue for SSNMR structural studies of membrane proteins. Most recently, we have developed a dual induction system in the SPP system, using amino acid auxotrophs enabling complete suppression of target protein production

before the addition of isotopes (Vaiphei et al. 2010). Combination of these technologies further makes the SPP system highly feasible for NMR protein structural studies.

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