Article

Cell-free protein synthesis in an autoinduction system for NMR studies of protein–protein interactions

Kiyoshi Ozawa[a], Slobodan Jergic[a], Jeffrey A. Crowther[a], Phillip R. Thompson[a], Gene Wijffels[b], Gottfried Otting[a] & Nicholas A. Dixon[a],*

[a]*Research School of Chemistry, Australian National University, Canberra, ACT 0200, Australia;* [b]*CSIRO Livestock Industries, Queensland Bioscience Precinct, St. Lucia, Queensland, 4068, Australia*

Abstract

Cell-free protein synthesis systems provide facile access to proteins in a nascent state that enables formation of soluble, native protein–protein complexes even if one of the protein components is prone to self-aggregation and precipitation. Combined with selective isotope-labeling, this allows the rapid analysis of protein–protein interactions with few ^{15}N-HSQC spectra. The concept is demonstrated with binary and ternary complexes between the χ, ψ and γ subunits of *Escherichia coli* DNA polymerase III: nascent, selectively ^{15}N-labeled ψ produced in the presence of χ resulted in a soluble, correctly folded χ-ψ complex, whereas ψ alone precipitated irrespective of whether γ was present or not. The ^{15}N-HSQC spectra showed that the N-terminal segment of ψ is mobile in the χ-ψ complex, yet important for its binding to γ. The sample preparation was greatly enhanced by an autoinduction strategy, where the T7 RNA polymerase needed for transcription of a gene in a T7-promoter vector was produced *in situ*.

Introduction

Cell-free protein synthesis using *Escherichia coli* S30 cell extracts is a powerful technique for the production of selectively isotope-labeled proteins for NMR spectroscopy, addressing one of the most grievous bottlenecks for protein NMR studies (Kigawa et al., 1999, 2004; Guignard et al., 2002; Klammt et al., 2004; Torizawa et al., 2004; Ozawa et al., 2004; Neerathilingam et al., 2005). Here we show the use of cell-free protein synthesis to form a soluble, correctly folded protein–protein complex with a protein that on its own is produced in an insoluble form both *in vivo* and *in vitro*. Complex formation from a nascent polypeptide chain under native conditions presents an attractive alternative to refolding protocols from urea solutions. We show that it can further be used for selective isotope labeling of one of the partners in the complex. In addition, an autoinduction strategy is introduced which simplifies cell-free protein synthesis and can greatly improve yields for protein products of genes that are expressed slowly.

The present study was carried out to investigate binary and ternary complexes among the χ (16.6 kDa), ψ (15.2 kDa) and γ (47.5 kDa) clamp-loader subunits of *E. coli* DNA polymerase III holoenzyme (Pol III). The crystal structure of the χ-ψ heterodimer showed no electron density for the N-terminal 26 residues of ψ (Gulbis et al., 2004), suggesting that this segment may be relatively flexible. The χ-ψ complex is known to bind to γ, which by itself exists as an equilibrium mix-

*To whom correspondence should be addressed. E-mail: dixon@rsc.anu.edu.au

ture of monomers and tetramers (Pritchard et al., 2000). The interaction is thought to be mediated by ψ, since χ alone does not bind to γ (Xiao et al., 1993a). Gulbis et al. (2004) hypothesized that the flexible N-terminal segment of ψ is required for binding to γ, resulting in its immobilization in the χ-ψ-γ complex. We addressed this question by preparation of χ-ψ complexes with selectively ^{15}N-Trp, ^{15}N-Phe and ^{15}N-Ala labeled ψ, respectively, to identify ^{15}N-HSQC peaks from the mobile N-terminal segment. Binding to γ was monitored by their disappearance upon addition of γ, and reappearance after addition of a synthetic peptide corresponding to the N-terminal 26 residues of ψ, $\psi(1$–$26)$, to the ternary χ-ψ-γ complex.

The ψ subunit is found in inclusion bodies when overproduced *in vivo* and precipitates during *in vitro* synthesis. Using cell-free protein synthesis, we achieved the formation of a soluble, correctly folded χ-ψ complex under non-denaturing conditions, where ψ was selectively labeled with ^{15}N-Ala. The presence of χ, however, exerted an inhibitory effect on the production of ψ so that NMR quantities of the χ-ψ complex were more efficiently prepared by refolding of ψ from urea solutions in the presence of separately purified χ as in the procedure established in earlier studies (Xiao et al., 1993b; Gulbis et al., 2004). Yet, the present results establish the feasibility of the production for NMR spectroscopy of protein–protein complexes, where one of the protein components is selectively isotope-labeled and produced in the presence of its binding partner. Availability of the autoinduction system presented here further extends the range of proteins that can be produced in high yields by cell-free protein synthesis.

Materials and methods

Materials

Purchased materials included L-[U$-^{15}$N]tryptophan and L-[^{15}N]phenylalanine (Cambridge Isotope Laboratories), L-[^{15}N]alanine (Spectra Stable Isotopes), RNase inhibitor (Promega), creatine kinase and *E. coli* total tRNA (Roche), Spectrapor-2 and -4 dialysis tubing (Spectrum Laboratories), the *E. coli* Rosetta^{TM} (λDE3)/pRARE strain (Novagen) and a 26-residue peptide, $\psi(1$–$26)$, with the sequence Biotin-MTSRRDWQLQQLGITQ-

WSLRRPGALQ-NH$_2$ (Auspep). *E. coli* BL21 (λDE3) and plasmid pLysS were as described (Studier et al., 1990). Plasmid pKO1166, which contains phage T7 *gene 1* (that encodes T7 RNA polymerase, RNAP) under transcriptional control of the phage λ p_L promoter, has been described (Ozawa et al., 2004). Plasmids pET-χ and pET-ψ, used for expression of the *E. coli holC* and *holD* genes, respectively (Xiao et al., 1993b), were generous gifts of Dr. Mike O'Donnell. The plasmid pJC490, used for overproduction of the γ subunit of Pol III, contained the *dnaX* gene downstream of tandem λ p_R and p_L promoters in vector pMA200U (Elvin et al., 1990). The gene had been provided with a strong ribosome-binding site and modified by oligonucleotide-directed mutagenesis to change codons 427–433 of *dnaX* to ensure production of γ but not τ (Tsuchihashi and Kornberg, 1990) (see online supplementary material for details).

Proteins

T7 RNAP was prepared as described by Ozawa et al. (2004). The χ subunit of Pol III was produced *in vivo* using strain BL21(λDE3)/pLysS/ pET-χ and purified essentially as described (Xiao et al., 1993b). The γ subunit was purified essentially to homogeneity from strain BL21(λDE3)/ pJC490, as described in the online supplementary material. Concentrations of proteins and the ψ (1–26) peptide were determined spectrophotometrically at 280 nm, using values of ε_{280} of 20340, 29160 and 11380 M^{-1} cm^{-1} for γ, χ and $\psi(1$–$26)$, respectively (Gill and von Hippel, 1989).

Cell-free protein synthesis

The S30 cell extract was prepared from the *E. coli* strain Rosetta (λDE3)/pRARE following the procedure of Pratt (1984), followed by concentration with polyethylene glycol 8000 (Kigawa et al., 1999). In our experience, S30 extracts concentrated in this way (S30K) resulted in about 30% higher yields of protein than a combination of a high-speed supernatant (S135) and a ribosomal fraction (Guignard et al., 2002), when expressing genes under control of the T7 promoter with purified T7 RNAP added to the reaction mixture. Furthermore, S30K extracts made from the Rosetta strain tended to yield more protein than those made with strain A19 (Kigawa et al., 2004). Note that the

Rosetta strain was not induced for expression of T7 RNAP prior to preparation of the extract.

For small-scale *in vitro* synthesis of unlabeled proteins, the inner-chamber reaction mixtures (total volume 100 μl) contained 55 mM HEPES/ KOH (pH 7.5), 1.7 mM dithiothreitol, 1.2 mM ATP, 0.8 mM each of CTP, GTP and UTP, 0.64 mM 3′,5′-cyclic AMP, 68 μM folinic acid, 27.5 mM ammonium acetate, 208 mM potassium glutamate, 80 mM creatine phosphate, 25 μg creatine kinase, 1 mM of each of the twenty L-amino acids, 15 mM magnesium acetate, 17.5 μg *E. coli* total tRNA, 18 μl of Rosetta (λDE3)/pRARE S30K extract, 1.6 μg of supercoiled plasmid DNA, 150 units of RNase inhibitor, and either 9.3 μg of purified T7 RNAP or 3.2 μg of supercoiled plasmid pKO1166. Since T7 RNAP contains 100 alanine residues, the concentration of L-Ala was doubled when using pKO1166. Cell-free protein synthesis was carried out in Spectrapor 2 dialysis tubing with a nominal size cutoff of 12–14 kDa for 8 h at 37 °C with gentle shaking in 3 ml of outer chamber solution. The inner chamber assembly and the outer chamber buffer were housed within a screw-capped 10 ml polypropylene tube. The outer chamber solution had the same composition as the inner chamber reaction mixture, except that S30K extract, tRNA, plasmid DNA, pKO1166 or T7 RNAP, creatine kinase and RNase inhibitor were omitted, and the concentration of magnesium acetate was increased to 19.3 mM. After incubation, the soluble and insoluble components of the inner chamber were separated by centrifugation (30,000×*g*, 1 h at 4 °C).

NMR analysis

Formation of soluble χ-ψ and γ-ψ complexes was investigated by synthesizing nascent ψ as described above in the presence of the χ (0.5–2.0 mg/ml) and γ (4–8 mg/ml) subunits, respectively. For NMR analysis, the χ-ψ complex was prepared by producing selectively ^{15}N-labeled ψ *in vitro* from plasmid pET-ψ with 2 mM ^{15}N-Ala, 50 μM ^{15}N-Trp, or 350 μM ^{15}N-Phe in place of the corresponding unlabeled amino acids, and the volumes of inner and outer chamber mixtures were increased to 0.7 and 7 ml, respectively. In contrast to earlier protocols (Kigawa et al., 1999; Guignard et al., 2002; Ozawa et al., 2004), the outside buffer was not exchanged during the reaction, as this did

not affect yields of ψ. After the end of the reaction and centrifugation, the pellet containing ψ was collected and solubilized with 0.2 ml of NMR buffer (20 mM Tris–HCl, pH 6.9, 0.5 mM EDTA, 0.2 M NaCl, and 2 M dithiothreitol) containing 6 M urea. This solution was mixed with separately purified χ (2.1 mg) in NMR buffer (total volume 3 ml) and dialyzed overnight in Spectrapor 4 tubing against 1 l of NMR buffer at 4 °C. The dialysate was centrifuged (30,000×*g*, 1 h at 4 °C) and the supernatant concentrated to a final volume of about 0.6 ml using Amicon Ultra-4 centrifugal filters (size cut-off 10 kDa). Three separate NMR samples of labeled ψ (typical concentration 0.1 mM) were prepared in this manner with ^{15}N-Ala, ^{15}N-Trp or ^{15}N-Phe. D$_2$O was added to a final concentration of 10% (v/v) before NMR measurements; samples were flash frozen in liquid nitrogen and stored at −70 °C until use.

In order to obtain a large quantity sufficient for NMR analysis of the ^{15}N-Ala-ψ-χ complex by cell-free production of ^{15}N-Ala-ψ (without refolding) in the presence of unlabeled χ, the production was carried out in parallel in eight separate reactions, each with 0.25 ml of reaction mixture and 7.5 ml of outside buffer. The mixtures were combined after dialysis into the NMR buffer, centrifuged and concentrated to a final volume of about 0.7 ml.

All NMR spectra were recorded at 25 °C using a Bruker Avance 800 MHz NMR spectrometer. ^{15}N-HSQC spectra were recorded using t_{1max} = 32 ms, t_{2max} = 146 ms and total recording times between 1.5 and 15 h per spectrum, except for the ^{15}N-HSQC spectrum of the ^{15}N-Ala-ψ-χ complex where ^{15}N-Ala-ψ had been prepared by cell-free synthesis with unlabeled χ present in the reaction mixture. This spectrum was recorded using a Varian INOVA 600 MHz NMR spectrometer using t_{1max} = 32 ms, t_{2max} = 102 ms and a total recording time of 23 h.
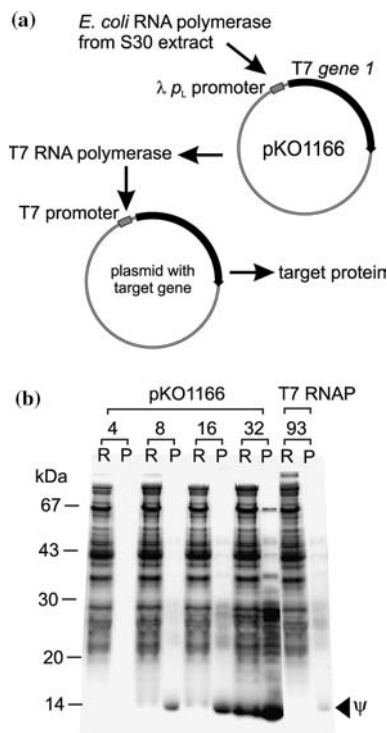
Results and discussion

For maximum yields in cell-free protein synthesis, additional RNA polymerase (RNAP) must be supplied for transcription (Kigawa et al., 1999; Guignard et al., 2002; Ozawa et al., 2004). Although increased T7 RNAP concentrations can be obtained by preparation of the S30 extract from an
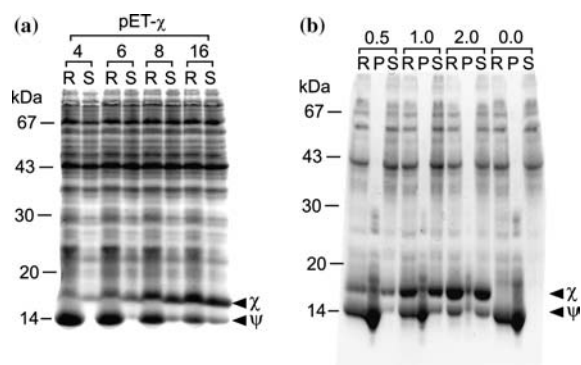
E. coli strain induced for overexpression of T7 RNAP (Nishimura et al., 1995), we show in the following that it is not only possible but also in some cases advantageous to co-synthesize T7 RNAP by transcription of its gene by the *E. coli* RNAP present in the S30 extract, in the manner of an autoinduction system (Figure 1a). The T7 *gene 1* that encodes RNAP is conveniently supplied by a second plasmid, such as pKO1166 (Ozawa et al., 2004). In the case of ψ, protein production directed by the pET-ψ plasmid was poor when T7 RNAP was supplied, but much higher protein yields were obtained by its co-synthesis (Figure 1b). We also tested the production of other proteins (PpiB, *h*CypA, and the Pol III subunits χ, ε and θ) and found the autoinduction method to work equally well or better (in the case of θ) than the original


Figure 1. (a) Scheme of the *in vitro* autoinduction system. The plasmids are expressed non-competitively, since their transcription depends on different RNA polymerases. (b) Comparison of the production of ψ using 16 μg/ml pET-ψ. *In vitro* synthesis reactions were carried out with increasing concentrations of pKO1166 or T7 RNAP (concentrations indicated in μg/ml). Proteins in equal proportions of the complete reaction mixtures (R) or the pelleted (P) fractions were separated by 15% SDS-PAGE. Mobilities of molecular mass markers were as indicated (in kDa).

systems with supplied *E. coli* or T7 RNAP (Ozawa et al., 2004). In general, protein production was maximal when pKO1166 was added at a final concentration of 16 μg/ml in the reaction chamber (data not shown). Only in the case of ψ (Figure 1b) and θ were yields improved by further increasing the concentration of pKO1166, whereas optimal production of PpiB and *h*CypA was already achieved with 8 μg/ml pKO1166. Addition of purified *E. coli* RNAP did not increase the expression levels, indicating that the amount of *E. coli* RNAP in the S30 extract was adequate for transcription of T7 *gene 1*. Production of T7 RNAP was not efficient enough to enable its easy detection by SDS-PAGE or to complicate production of labeled samples, but was clearly sufficient to transcribe target genes. Compared to a recently proposed scheme, where the T7 RNA polymerase gene was located on the same vector as the gene of the protein of interest (Ishikawa et al., 2004), our autoinduction system offers (i) greater control of the effective T7 RNA polymerase concentration and (ii) the use of conventional unmodified T7-promoter vectors as used for *in vivo* protein production.

Similar to the situation *in vivo* (Xiao et al., 1993b; Gulbis et al., 2004), ψ was produced in insoluble form in the *in vitro* system. The advantage of the autoinduction system for the production of ψ appeared to be related to the comparatively slow rate of expression of its gene (in pET-ψ), since co-production of PpiB and ψ resulted in overwhelmingly dominant production of PpiB (data not shown). Co-expression of *holC* (encoding χ) and *holD* (encoding ψ) also resulted in higher yields of χ than ψ (using pET-χ, pET-ψ and pKO1166 at 16 μg/ml; increased levels of pKO1166 up to 48 μg/ml did not change this effect). The yield of ψ increased substantially when the amounts of pET-χ were reduced (Figure 2a). In the case of χ and ψ, however, the production of ψ was also actively inhibited by the presence of χ protein (Figure 2b). Notably, when ψ was made with χ present, increasing the amount of χ resulted in a greater portion of ψ in the soluble fraction as a result of formation of the soluble χ-ψ complex (Figure 2b); χ thus captured the nascent ψ protein and prevented its precipitation. In contrast, however, cell-free synthesis of ψ in the presence of γ did not lead to production of a soluble γ-ψ complex.

Figure 2. (a) Co-expression of ψ (from pET-ψ at different concentrations of pET-χ (indicated in μg/ml). Alternate lanes show the complete reaction mixtures (R) and the soluble (S) fractions separated by 15% SDS-PAGE. (b) Expression of ψ (from pET-ψ in the presence of protein χ (concentrations in mg/ml). The lanes show the reaction mixtures (R), pelleted (P) and soluble fractions (S).

The system was used for the production of a χ-ψ complex in which ψ was selectively labeled with ^{15}N-Ala. Due to the specific inhibitory effect of χ on the production of ψ, the yields were low and the cell-free reaction had to be scaled up to obtain sufficient protein for analysis by NMR spectroscopy. The dispersion of chemical shifts observed in the ^{15}N-HSQC spectrum (Figure 3a) indicated that ψ had assumed a defined three-dimensional fold. An indistinguishable ^{15}N-HSQC spectrum was obtained with a sample produced by reconstitution of the χ-ψ complex by combining purified χ with urea-solubilized ^{15}N-Ala-labeled ψ made separately by cell-free synthesis (Figure 3b). This shows that, in the presence of χ, nascent ψ folded into the same structure as ψ that had been refolded under equilibrium conditions from urea-solubilized protein. This structure must be the native, correctly folded structure, since our reconstitution method was the same as that used to prepare the χ-ψ complex for structure determination by X-ray crystallography (Gulbis et al., 2004). Because of the improved yields obtained by reconstitution of the χ-ψ complex from urea-solubilized ψ, we used this procedure for the preparation of all subsequent NMR samples of the χ–ψ complex.

The observation of narrow and intense NMR signals provides a sensitive criterion for increased mobility of polypeptide chains on the nanosecond time scale. Using ^{15}N-labeling, NMR signals from the corresponding backbone amide groups can be
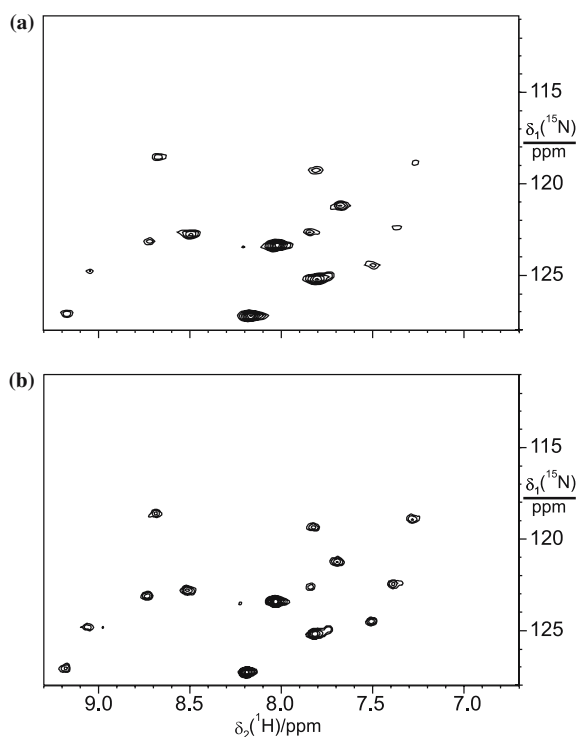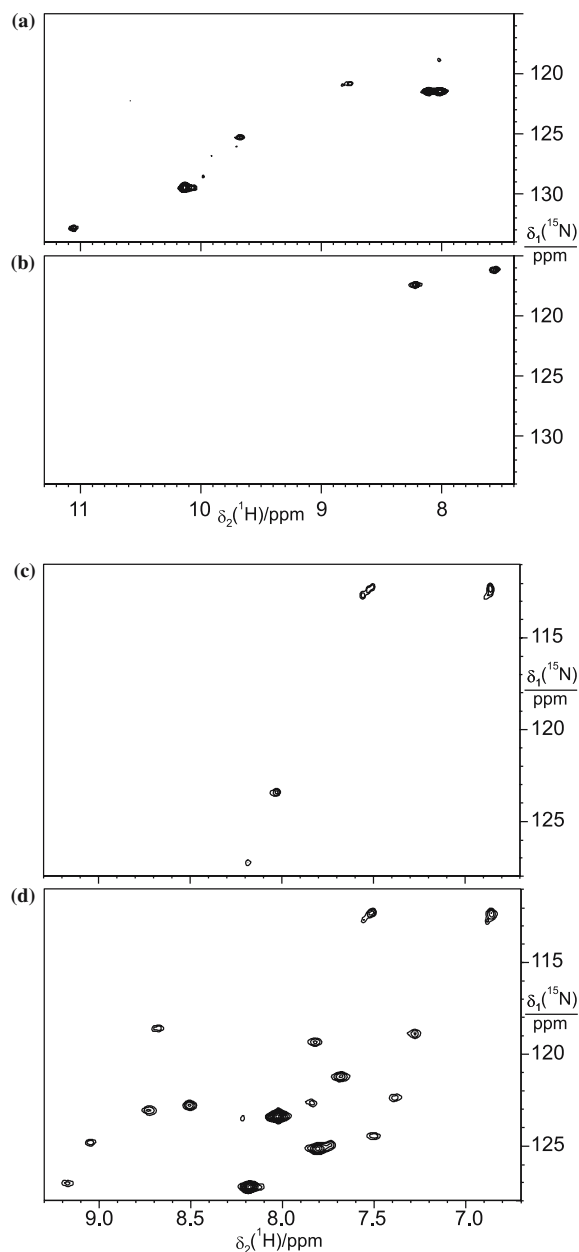

Figure 3. ^{15}N-HSQC spectra of selectively ^{15}N-Ala-labeled ψ in complex with unlabeled χ. The spectra were recorded at pH 6.9 and 25 °C. (a) Spectrum recorded of the ψ-χ complex prepared by cell-free synthesis of ^{15}N-Ala-ψ in the presence of χ. The spectrum was recorded in 23 h on a 600 MHz NMR spectrometer. (b) Spectrum recorded of the ψ-χ complex prepared by reconstitution of the complex from urea-solubilized ^{15}N-Ala-ψ. The spectrum was recorded in 1.5 h on an 800 MHz NMR spectrometer. The similarity between these NMR spectra indicates that the same three-dimensional fold of ψ is obtained by both procedures.

selectively detected in ^{15}N-HSQC spectra without prior separation of the ^{15}N-labeled protein from the reaction mixture (Guignard et al., 2002). We used the peak heights observed in the ^{15}N-HSQC spectra to assess the mobility and, hence, structural significance of the N- or C-terminal ends of ψ in the χ-ψ complex. Sequence-specific resonance assignments of ψ are not available. Therefore, the mobility of the terminal ends was investigated by the use of three samples of ψ, selectively labeled with ^{15}N-Ala, ^{15}N-Trp or ^{15}N-Phe, respectively. Cross-peaks for all 15 Ala residues in ψ could be observed, showing that the sensitivity was sufficient to observe signals over the entire length of ψ in the 32 kDa χ-ψ complex (Figure 3). Only one of the alanines in ψ is located in the N-terminal segment (at position 24) as are two of the four

tryptophans (at position 7 and 17), whereas both phenylalanines are located near the C-terminus (at position 132 and 133; ψ contains 137 residues in total). As expected for increased mobility of the N-terminal segment, two of the backbone amide protons of Trp yielded intense ^{15}N-HSQC cross-peaks (near 8 ppm; Figure 4a) that were observable already in an experiment recorded in 1.5 h. In contrast, the cross-peaks from Phe (Figure 4b) were observable only in an overnight experiment, indicating that these residues are not in a mobile segment.

During the recording of the NMR spectra of the χ-ψ complexes, some precipitate formed. Analysis by SDS-PAGE showed that the precipitate contained only (excess) χ, i.e., all ψ remained in solution in the χ-ψ complex. Thus, ψ stabilized χ against precipitation just as χ prevented ψ from aggregation.

Solutions of γ subunit of Pol III were added to the χ–ψ complexes containing ^{15}N-Ala-ψ and ^{15}N-Trp-ψ, and ^{15}N-HSQC spectra were recorded. Binding to γ was evidenced by the disappearance of the cross-peaks from ψ (Figure 4c; the residual signal intensity from ^{15}N-Ala-ψ (Figure 3b) is explained by a substoichiometric quantity of γ). The new signals near 112 ppm (^{15}N) seem to stem from highly mobile NH$_2$ groups of γ at natural isotopic abundance. The same result was obtained with ^{15}N-Trp–ψ, except that all signals from ψ disappeared completely (data not shown). The ternary χ-ψ-γ complex has a molecular weight of over 120 kDa, if γ is assumed to be at least dimeric in the complex (Xiao et al., 1993a; Pritchard et al., 2000). T_2 relaxation time measurements by ^{1}H spin-echo experiments were consistent with a M_r of about 150 kDa (data not shown). This high value explains the disappearance of ^{15}N-HSQC signals from ψ. If the N-terminal segment of ψ were still highly mobile in the χ-ψ-γ complex, however, its ^{15}N-HSQC cross-peaks ought still to have been observable. The N-terminal segment of ψ thus appears to be immobilized in the χ–ψ-γ complex, suggesting that it contributes to the binding interface. This result was further supported by the observation that the NMR spectrum of ^{15}N-Ala-ψ in the χ-ψ complex reappeared after addition of the ψ(1–26) peptide (Figure 4d). Although the peptide was added in two-fold excess over ψ, the signals of the χ-ψ complex were weaker than in the absence of γ (Figure 3b), indicating that not all of the χ-ψ complexes

Figure 4. ^{15}N-HSQC spectra of selectively ^{15}N-labeled ψ in binary and ternary complexes with χ and γ recorded at pH 6.9 and 25 °C. The ψ-χ complexes were prepared by refolding from urea-solubilized ψ. Each spectrum took between 13 and 15 h to record. (a) ^{15}N-Trp–ψ in complex with χ. Backbone and side-chain nitrogens of Trp were labeled. (b) ^{15}N-Phe-ψ in complex with χ. (c) ^{15}N-Ala-ψ in complex with χ (same sample as in Figure 3b) after addition of γ to a final concentration of 0.16 mM (as monomer). (d) Same as (c), after addition of ψ(1–26) to a final concentration of 0.24 mM.

had been released from γ. Therefore, the N-terminal segment of ψ confers part, but perhaps not all, of its affinity of binding to γ.

Conclusions

The *in vitro* autoinduction system appears to be generally applicable for the expression of genes under control of the T7 promoter. In all the examples tested in our laboratory, it provided similar or higher protein yields than corresponding systems based on purified *E. coli* or T7 RNAP (Guignard et al., 2002; Ozawa et al., 2004). The autoinduction protocol is easy to use and may favor more slowly expressed genes by generating a continuous supply of T7 RNAP which, due to the limited stability of T7 RNAP under the reaction conditions, could not be achieved by provision of T7 RNAP at the start of the reaction (Nishimura et al., 1995; Ishikawa et al., 2004). Purified RNA polymerases are the most expensive components of cell-free reaction systems and their expression and purification can be cumbersome without appropriate equipment. The autoinduction protocol replaces them by the use of a plasmid that can easily be made in large quantities using commercial kits.

The present study showed that nascent ψ was rescued from precipitation by binding to χ when χ was present in the mixture. In addition, the structure of ψ formed in this way was the same as the structure obtained by the refolding protocol used for crystal structure determination, indicating that the protein was correctly folded in a native conformation. Cell-free protein synthesis thus presents an excellent method to build and detect complexes between marginally stable proteins without the need for refolding from denaturing conditions. Combined with the possibility of selective isotope labeling of one of the protein components and NMR analysis of the mobility of terminal polypeptide chains, cell-free protein synthesis carries a unique potential for the study of protein–protein interactions.

Acknowledgements

Supplementary material to this paper is available in electronic format at http://dx.doi.org/10.1007/s10858-005-7946-4. It describes the overexpression and purification of the γ subunit of *E. coli* DNA polymerase III.

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