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In vitro expression and characterization of native apomyoglobin under low molecular crowding conditions

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Abstract

The labile nature of membranes and organelles poses serious challenges to in situ biomolecule characterization in intact cells. Cell-free in vitro systems provide an alternative promising medium for the expression and characterization of protein conformation and function in a biochemical context that bears several similarities to the cellular environment. In addition, cell-free transcription–translation has recently emerged as a convenient method for protein selective isotope labeling, providing significant advantages for detailed NMR analysis. We report the cell-free expression of the model protein apomyoglobin (apoMb) in an *Escherichia coli* cell-free system and the effect of polyethylene glycol (PEG) on the expression yields. In contrast with in vivo protein production under control of the strong T7 promoter, apoMb is expressed in vitro in 100% soluble form. In-gel tryptic digestion followed by mass spectrometry were performed to confirm the protein identity. In order to probe the conformation of the newly expressed protein and investigate the feasibility of in situ structural analysis, high resolution protein characterization was carried out by 2D NMR spectroscopy. In vitro apoMb expression in a PEG-free environment is a convenient method for the production of soluble native-like protein under conditions amenable to selective isotopic labeling. Yields can be easily scaled-up by dialysis-assisted cell-free expression.
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Cell-free expression has gained considerable interest as an effective alternative to in vivo protein production. This is largely due to significant improvements in the batch and continuous action (i.e., dialysis and bioreactor-based) modes of cell-free transcription–translation [1–4]. Much of the driving force for the above methodological advances has been stimulated by the structural biology and proteomics communities, which have exploited the unique advantages offered by cell-free expression for the production of selectively (and uniformly) labeled proteins for NMR applications [5–11], the synthesis of selenomethionine-labeled proteins for X-ray crystallography [8], and efficient protein expression for high-throughput screening and selection [12–15].

Protein expression for functional and structural genomics applications requires the efficient sampling of multiple experimental conditions. The cell-free platform provides an ideal medium for parallel screening and expression array setups, given the typically small expression volumes and the ability to easily add different expression components.

Another area where cell-free expression is starting to have a significant impact is the biophysical investigation of protein behavior in environments bearing some resemblance to those of living cells. For instance, fundamental biological phenomena such as protein stability, protein folding, and protein–protein interactions are best studied in a physiologically relevant context. Cell-based investigations of the above events, however, are

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complicated by the unfavorable response of most available spectroscopic tools to the heterogeneous nature of living cells. In addition, the labile character of the cell wall, membranes, and organelles, which are known to often undergo spontaneous lysis if not properly treated, poses serious challenges to performing in situ investigations within intact cells. Cell-free systems contain a large fraction of the intracellular protein-based machinery of a cell and are devoid of complications due to the presence of membranes or other labile components. Therefore, cell-free systems are emerging as a promising medium for the characterization of protein conformation and function in a biochemical context similar to that of the cellular environment. In order to perform biophysical investigations in cell-free systems, the target protein must be successfully expressed in soluble form. In addition, it must be selectively detectable within the pool of cell-free proteins. This is efficiently achieved by cell-free expression coupled with selective tagging by either isotopically enriched amino acids, spin labels or fluorescent labels. We show here that an *Escherichia coli* cell-free system lacking polyethylene glycol (PEG)[1] bears special advantages for both the analytical characterization and conformational analysis of the model all-α-helical protein apomyoglobin (apoMb).

Preparation of *E. coli* cell-free systems is relatively straightforward and well documented in the literature [5,16–19]. In addition, cell-free protein expression kits are available from several commercial sources. In vitro expression results are typically analyzed by sodium dodecyl sulfate gel electrophoresis (SDS–PAGE) followed by Western blot, autoradiography, or direct staining with Coomassie blue (provided expression yields are sufficiently high). Certain components of typical cell-free reaction mixtures, such as PEG, are known to interfere with detection by altering protein migration behavior in specific regions of the gel. In standard *E. coli* cell-free preparations, interference from PEG prevents the direct detection of proteins smaller than ∼30 kDa by gel electrophoresis. Therefore, typical procedures for the analysis of cell-free protein expression include a step targeted at the removal of potentially interfering components. Fast and efficient analysis, however, requires a more streamlined approach.

This report shows that apoMb can be efficiently expressed in an in vitro *E. coli* cell-free system under low molecular crowding conditions, i.e., in a PEG-free environment. The absence of PEG simplifies analytical identification by gel and Western blot. The protein is conveniently produced in 100% soluble form in its native state. This is a considerable advantage over the corresponding in vivo expression (utilizing the same vector), which leads to partially or totally insoluble protein. The method presented here leads to the concurrent simplification of apoMb detection, screening of in vitro expression conditions, purification, and in situ biophysical characterization.

Materials and methods

Materials

Amino acids were purchased from Advanced ChemTech (Louisville, KY). Nucleotides were obtained from Roche Applied Science (Indianapolis, IN). T7 RNA polymerase was purchased from Ambion (Austin, TX). L-[^{35}S]Methionine (1175 Ci/mmol) was obtained from Perkin-Elmer (Shelton, CT). Dialysis membrane (12–14 kDa molecular weight cutoff, MWCO) was purchased from Spectrum Laboratories, (Rancho Dominguez, CA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Plasmid constructs

The original plasmid containing the sperm whale apomyoglobin (apoMb) gene (optimized for translation in *E. coli* [20]) was provided by Dr. Stephen Sligar. The apoMb gene was subcloned into a pET-17b vector (Novagen, Madison, WI) and inserted between the *Nde*I and *Kpn*I restriction sites. A second apoMb plasmid was created for antibody-based Western blot detection. This plasmid encodes for an N-terminal 11-amino acid fusion peptide (T7-tag) immediately preceding the apoMb sequence. This T7-tagged apoMb construct was obtained by subcloning the apoMb gene into a different position on the pET-17b vector, between the *Hin*dIII and *Kpn*I restriction sites. Plasmids containing the genes for streptococcal protein G (Gb1) and *E. coli* cold shock protein A (CspA) were generously supplied by Dr. Kevin Gardner and Dr. Masayori Inouye, respectively. The plasmid containing the chloramphenicol acetyltransferase (CAT) gene was obtained as the positive control test plasmid in the PROTEINscript-PRO kit (Ambion, Austin, TX). All target gene sequences are under the control of the T7 promoter.

Cell-free system preparation and protein expression

The cell-free system used for protein synthesis was prepared from *E. coli* strain A19 according to known methods [17] with the following modifications. Cells for

[1] *Abbreviations used:* apoMb, *Sperm whale* apomyoglobin; CAT, chloramphenicol acetyltransferase; CspA, cold shock protein A; Gb1, streptococcal protein G (B1 domain); NC, negative control; PEG, polyethylene glycol; MWCO, molecular weight cutoff; DTT, dithiothreitol; TCA, trichloroacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; BME, 2-mercaptoethanol; MS/MS, tandem mass spectrometry.

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