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Addition of negatively charged residues can reverse the decrease in the solubility of an acidic protein caused by an artificially introduced non-polar surface patch

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ABSTRACT

A non-polar patch on the surface of a protein can cause a reduction in the solubility and stability of the protein, and thereby induce aggregation. However, a non-polar patch may be required so that the protein can bind to another molecule. The mutant 6L—derived from the acidic, dimeric α-helical protein sulerythrin and containing six additional leucines arranged to form a non-polar patch on its surface when properly folded—has a substantially reduced solubility in comparison with that of wild-type sulerythrin. This reduced solubility appears to cause 6L to aggregate. To reverse this aggregation, we mutated 6L so that it contained three to six additional glutamates or aspartates that we predicted would surround the non-polar leucine patch on natively folded 6L. Although the introduction of three glutamates or aspartates increased solubility, the mutants still aggregate and have a reduced α-helical content. Conversely, mutants with six additional glutamates or aspartates appear to exist mostly as dimers and to have the same α-helical content as that of wild-type sulerythrin. Notably, the introduction of five lysines or five arginines at the positions held by the glutamates or aspartates did not recover solubility as effectively as did the negatively charged residues. These results demonstrate that negatively charged residues, but not positively charged ones, surrounding a non-polar patch on an acidic protein can completely reverse the decrease in its solubility caused by the patch of non-polar surface residues.

1. Introduction

The non-polar portions of the side chains of residues that reside on the surface of a protein in aqueous medium often interact with other molecules, including other proteins [1–3], membranes [4,5], and small-molecule ligands [6–8] that also contain non-polar surface regions. The hydrophobic effect, involving a gain in entropy when a non-polar moiety is removed from the aqueous medium, drives the aforementioned interactions [9], and has been used to bind proteins onto an artificial surface [10], carbon nanotubes [11], and fullerenes [12]. Dieckmann and colleagues designed a peptide that increases the dispersion of carbon nanotubes in water by interacting with their surfaces [13]. Fleishman and colleagues created a protein that mimics an antibody in that it binds to the influenza virus hemagglutinin and thereby inhibits the hemagglutinin conformational change necessary for the uptake of the virus by cells lining the respiratory tract [14]. Therefore, introduction of an artificial non-polar patch on the surface of a protein may be used to promote an interaction with another molecule that will have a practical application. However, a non-polar surface artificially introduced into an otherwise soluble protein may decrease its solubility [15] causing it to unfold and/or aggregate [16,17].

For the study reported herein, we first created 6L—a mutant of the soluble, acidic protein sulerythrin from the hyperthermophilic Archaeon *Sulfolobus tokodaii*—that would have a non-polar patch on its surface if folded natively. Native sulerythrin is an extremely thermostable eight-helix bundle containing two polypeptide chains. Because introducing a non-polar patch on a protein surface is often destabilizing, we wished to use an intrinsically stable protein. Sulerythrin has two helices aligned in parallel on which a large (designed) non-polar patch might serve as a binding platform for a second macromolecule. Moreover, when introducing a non-polar patch onto the surface of a protein and having the choice of two or more proteins, inherently, the protein of greatest molecular mass would be the preferred scaffolding because the ratio of its non-polar patch surface area to the remainder of the (presumably) hydrophilic surface area would be the smallest for the proteins under consideration. Given these observations, sulerythrin is likely to provide a more suitable platform than are leptin and RNase Sa, for which solubility has been improved by the introduction of charged surface residues [18,19].

After finding that 6L aggregated, to decrease the apparent nonspecific hydrophobic interactions between 6L molecules, which probably caused it to aggregate, we introduced three to six glutamates or aspartates that would surround the non-polar patch in natively folded 6L in an attempt to reverse the aggregation. Our design principle incorporated the observations that repulsion by negatively charged surface residues counters

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the hydrophobic effect induced by non-polar surface residues [20,21]. We then assessed how the solubility, degree of intermolecular association, and the secondary and tertiary structures of 6L are affected by the additional negative charges. Introduction of six aspartates most efficiently reversed the effect of the six leucines, i.e., the solubility of the protein increased, and aggregation and partial unfolding of the protein decreased substantially. Thus, the introduction of negatively charged surface residues into this acidic protein counteracts the effects on solubility induced by the non-polar surface patch.

2. Materials and methods

2.1. Construction of expression vectors for wild-type sulerythrin and its mutants

To construct expression vectors for wild-type sulerythrin and its mutants (with the exception of those in the 4L2A-derived library, see Section 2.3), first the gene encoding sulerythrin was PCR amplified from *S. tokodaii* genomic DNA and then cloned into the *Nde*I-*Hin*dIII site of a pET21c vector (Merck). The mutated genes were prepared by splicing-by-overlap-extension PCR [22] using the wild-type gene as the template. Each mutated gene was cloned into the *Nde*I-*Hin*dIII site of a pET21c vector. The nucleotide sequence encoding Gly–Gly–Cys, which allows proteins to be immobilized onto gold particles, was placed immediately downstream of each gene for future use.

2.2. Expression of WT sulerythrin and its mutants, and characterization of their solubilities in whole cell lysates

Expression plasmids for wild-type sulerythrin and its mutants, prepared as described in Section 2.1, were individually transformed into *Escherichia coli* Rosetta 2(DE3) cells, and the transformants were cultured at 37 °C for 12 h in 5 mL of Luria Broth, 150 μg/mL ampicillin. An *E. coli* Rosetta 2(DE3) inoculum harboring an empty pET21c was also cultured and served as the negative control. Protein expression was induced by addition of isopropyl-thio-β-D-galactopyranoside (IPTG; final concentration, 1 mM), after which the cells were cultured at 37 °C for an additional 6 h. The cells harboring expression plasmids for the sulerythrin mutants, 6L, 6L5K, and 6L5R were also cultured at 25 °C for 12 h after addition of IPTG to examine if the culture temperature affected the amount of soluble protein recovered. The cells were then harvested by centrifugation at 3000 ×*g* for 10 min, suspended in 200 μL of 20 mM Tris–HCl, pH 8.0, and sonicated. The resulting lysates were each separated into soluble and insoluble (precipitated) fractions by centrifugation at 20,000 ×*g* for 20 min. For visualization of the proteins, samples were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting with an anti-sulerythrin polyclonal antibody. An anti-rabbit IgG antibody labeled with alkaline phosphatase was used as a second antibody for visualization.

2.3. Preparation and screening of a library of arginine-containing 4L2A mutants to assess the ability of positively charged residues to increase solubility when surrounding a non-polar surface patch

The 4L2A gene, created as described in Section 2.1, was used as the template to create the plasmid library. Residues at positions 97, 102, 109, 113, 114, 121, 127, and 128 were subjected to semi-random mutagenesis. The codon for residue 97 was AGA or ACC; the codon for residue 102 was GGA or CGA; the codon for residue 109 was AGA or GAA; the codon for residue 113 was CGA or CCA; the codon for residue 114 was AGA or GAA; the codon for residue 121 was AGA or ACT; the codon for residue 127 was AAG or AGG; the codon for residue 128 was AGA or AGT. *E. coli* Rosetta 2(DE3) cells were transformed with the plasmid library, and then 380 isolated colonies were separately cultured in Luria Broth, 150 μg/mL ampicillin. Each mutant had a 74% probability of being present as one of the 380 clones. After induction with IPTG (final concentration, 1 mM), cells were harvested by centrifugation at 1000 ×*g* for 30 min and lysed using BugBuster Protein Extraction reagent (Novagen). The bacterial lysates were separated into soluble and insoluble fractions by centrifugation at 7000 ×*g* for 30 min at 4 °C, followed by SDS-PAGE.

2.4. Protein expression and purification

For the expression of wild-type sulerythrin and each soluble mutant, an *E. coli* Rosetta 2(DE3) inoculum harboring the appropriate plasmid was cultured at 37 °C for 3 h in 2L of Luria Broth, 150 μg/mL ampicillin. Expression was induced by addition of IPTG (final concentration, 1 mM). At the end of an additional 6-h culture, cells were harvested, suspended in 30 mL of 20 mM Tris–HCl, pH 8.0, and sonicated. Each cell lysate was treated with 25 units of benzonase (Novagen) for 1.5 h at 37 °C and then incubated at 70 °C for 30 min to precipitate *E. coli* proteins. Each soluble fraction was isolated by centrifugation at 40,000 ×*g* and 4 °C for 20 min, and then individually loaded onto a HiTrap Q HP column (GE Healthcare) for recovery of each flow-through fraction. To incorporate ferrous ion into the proteins, the flow-through fractions were dialyzed against 20 mM Tris–HCl, pH 8.0, 100 μM $FeSO_4$, 100 μM $ZnSO_4$, 2-mercaptoethanol at 70 °C for 1.5 h and then against 20 mM Tris–HCl, pH 8.0. Each dialyzed solution was loaded onto a Superdex 200 gel filtration column (GE Healthcare), and the appropriate fractions were collected and pooled. All purified proteins were homogeneous as judged by SDS-PAGE. Protein concentrations of the solutions were determined using their A_{280} values and an extinction coefficient of 18,450 M^{-1} cm^{-1} [23].

2.5. Analytical gel-filtration chromatography

Each protein (7.5 μM) was individually chromatographed at a flow rate of 0.5 mL/min through a Superdex 200 column that had been equilibrated with 20 mM Tris–HCl, pH 8.0, 150 mM NaCl; 20 mM $NaHCO_3$, pH 10, 150 mM NaCl; 20 mM sodium phosphate, pH 6.5, 150 mM NaCl; 20 mM Tris–HCl, pH 8.0, 500 mM NaCl; or 20 mM Tris–HCl, pH 8.0, 150 mM KCl. Apparent molecular masses were determined by comparing the protein elution volumes to those of a calibration curve that had been produced using proteins of known molecular mass and elution volume.

2.6. Circular dichroism (CD) spectroscopy

All CD spectra were recorded using a Jasco J720C spectropolarimeter. Far-UV CD spectra were used to determine the secondary structure content of each protein (7.5 μM protein in 20 mM sodium phosphate, pH 8.0, 150 mM NaCl). The spectra were recorded from 200 to 250 nm at 25 °C and with a cell of 0.1-cm path length. Near-UV CD spectra of the proteins (15 μM protein in 20 mM Tris–HCl, pH 8.0) were recorded from 250 to 320 nm at 25 °C and with a cell of 1.0-cm path length to assess the tertiary structures of the proteins. Spectra of far-UV and near-UV CD were collected at 0.1 nm intervals and each spectrum was the average of 20 scans.

2.7. 8-Anilino-1-naphthalenesulfonic acid (ANS) fluorescence spectroscopy

Samples of wild-type sulerythrin, 6L5E, 6L6E, 6L5D, and 6L6D each containing 25 μM ANS (0.5 μM protein in 20 mM sodium phosphate, pH 8.0) were held for 60 min in the dark. All fluorescence spectra were recorded from 450 to 600 nm with excitation at 375 nm using a Shimadzu RF-5300PC spectrofluorophotometer.