# The Physical Stability of the Recombinant Tuberculosis Fusion Antigens H1 and H56

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**ABSTRACT:** The recombinant fusion proteins hybrid 1 [H1 (Ag85B-ESAT-6)] and hybrid 56 [H56 (Ag85B-ESAT-6-Rv2660c)] derived from *Mycobacterium tuberculosis* are promising antigens for subunit vaccines against tuberculosis. Both antigens are early batches of antigens to be enrolled in human clinical trials and it is therefore important to characterize their conformational stability in solution as well as upon interaction with adjuvants. In this study, the physical stability of the two antigens was characterized using a number of biophysical techniques. Dynamic light scattering and sodium dodecyl sulfate–polyacrylamide gel electrophoresis analyses demonstrated that both antigens exist as a distribution of multimeric states under nonstressed conditions. Their conformational stability was monitored as a function of pH and temperature and visualized in three-index empirical phase diagrams. Both antigens showed a gradual loss of secondary as well as tertiary structure as a function of temperature, with no cooperative transitions observed. Preformulation studies with the Th1-inducing cationic adjuvant CAF01 showed that the antigens were almost completely surface adsorbed. Upon adsorption, the liposome size increased; however, the physical stabilities of the bound and the unbound antigens were comparable. This study provides important information about the biophysical properties of H1 and H56 and highlights the analytical challenges of characterizing complex vaccine formulations. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

**Keywords:** biopharmaceutical characterization; fluorescence spectroscopy; vaccine delivery; vaccine adjuvants; liposome

## INTRODUCTION

The emergence of extensively drug-resistant *Mycobacterium tuberculosis* strains emphasizes the need for medical strategies to prevent the global killer tuberculosis (TB).[1] The only approved vaccine against *M. tuberculosis* employs Bacillus Calmette–Guérin (BCG) and was introduced almost a century ago. Although the use of this vaccine is widespread and effective in children, it fails to provide significant protection for adults and adolescents.[2] There is, therefore, an unmet medical need for the development of more efficacious TB vaccines.

A promising vaccination strategy to prevent TB in adults and adolescents includes boosting BCG with subunit vaccines, based on one or more recombinant protein antigens derived from *M. tuberculosis*. These include the Ag85 family of antigens, ESAT-6 and CFP-10.[3] Mixtures of several proteins coadministered in one vaccine are challenging to develop. The use of fusion proteins, however, permits the inclusion of multiple antigens in a single recombinant polypeptide.[4–7] One such fusion protein, designated Ag85B-ESAT-6 [hybrid 1 (H1)], is composed of Ag85B (Rv1886) and ESAT-6 (Rv3875) (Table 1). Both proteins are secreted during the acute/early stage of infection, and have an extensive history as TB vaccine candidates.[3]

Latent TB infections often go unnoticed by the host until active TB is manifested.[2] Thus, the use of acute stage-secreted antigens may not be sufficient to enhance long-term multistage protection against TB. Therefore, the multistage fusion Ag85B-ESAT-6-Rv2660c [hybrid 56 (H56)] has been designed that contains the latency-associated antigen Rv2660c and H1 (Table 1).[8,9]

Proteins by themselves are usually poorly immunogenic, and coadministration with Th1-inducing adjuvants is necessary to develop efficacious TB vaccines.[3,10] Both H1 and H56 are currently tested in preclinical and clinical trials in combination with adjuvant systems which induce strong cell-mediated immune responses (NCT00922363, NCT01049282, NCT01003093, and NCT00929396).[8,9,11–14] One of those is CAF01, a cationic liposomal adjuvant [Statens Serum Institut (SSI), Copenhagen, Denmark], which is based on the cationic surfactant dimethyldioctadecylammonium (DDA) bromide and the immunopotentiator α,α′-trehalose 6,6′-dibehenate (TDB).[15] The cationic liposomes form a depot at the site of injection and TDB potentiates a strong $CD4^+$ T-cell response characterized by a mixed Th1/Th17 profile *via* interaction with the Mincle receptor on antigen-presenting cells.[12,16] These attributes make CAF01 a promising TB adjuvant.

Despite their clinical relevance, the structural and biophysical properties of H1 and H56 remain to be reported. The two recombinant fusion antigens represent an interesting class of proteins because their design is based on a specific desired immunogenicity through recognition of linear T-cell epitopes, often without taking the structural properties of the constructs into account. This makes a subsequent understanding of their

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**Table 1.** Composition, Molecular Weight, Calculated p*I*, Number of Tryptophan, and Cysteine Residues of the Fusion Proteins H1 and H56

| | Epitope Constructs | MW (g/mol) | Calculated p*I*[a] | Trp Residues | Cys Residues |
|---|---|---|---|---|---|
| H1 | Ag85B, ESAT-6 | 41119 | 5.0 | 13 | 3 |
| H56 | Ag85B, ESAT-6, Rv2660c | 48340 | 4.9 | 14 | 4 |

[a] p*I* was calculated using www.scripps.edu/~cdputnam/protcalc.html

physical stability, both in solution and in the presence of an adjuvant, crucial to ensure optimal vaccine stability.

Prior knowledge from the downstream processing and preformulation of H1 and H56 identified solubility and aggregation of the purified antigens as major challenges for their development into vaccine candidates. To improve the understanding of this behavior, the biophysical properties of early clinical batches of the two fusion proteins H1 and H56 were examined, both in solution and upon adsorption to the adjuvant CAF01. The conformational stability of the antigens was studied as a function of pH and temperature using circular dichroism (CD), intrinsic and extrinsic fluorescence, as well as static light scattering. The resultant data were combined to generate three-index empirical phase diagrams (EPDs) to provide a global picture of their solution behavior.[17]

## MATERIALS AND METHODS

### Materials

The recombinant protein antigens Ag85B-ESAT-6 (H1) and Ag85B-ESAT-6-Rv2660c (H56) were provided by SSI. Both proteins were expressed in *Escherichia coli* and the purity of the antigens was >95%, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, European Pharmacopoeia 2.2.31). The CAF01 liposomes composed of 2.5 mg/mL DDA (3.96 mM) and 0.5 mg/mL TDB (0.51 mM) corresponding to a DDA–TDB molar ratio of 89:11 prepared in 10 mM Tris buffer (pH 7.4) were kindly provided by SSI. Citric acid, dibasic sodium phosphate, Tris base, and sodium chloride were purchased from Fisher Scientific (Pittsburgh, Pennsylvania), and glycine was acquired from Bio-Rad (Hercules, California). All buffers were prepared using purified water (Labconco WaterPro PS, Kansas City, Missouri).

### Sample Preparation

H56 was provided in a 20 mM glycine buffer (pH 9.2), whereas H1 was provided in a 25 mM Tris + 10% glycerol buffer (pH 8.0). To standardize the conditions under which the stability was investigated, buffer exchange was performed using Slide-A-Lyzer dialysis cassettes (3.5 kDa molecular weight cutoff, Pierce, Rockford, Illinois) overnight at 5°C. H56 was dialyzed against 20 mM citrate phosphate (CP) buffer in the pH range of 5.5–8.0, at pH intervals of 0.5. Below pH 5.5, H56 was not soluble. H1 was dialyzed against 20 mM CP buffer in the pH range of 7.0–8.0, at pH intervals of 0.5, and against 20 mM glycine buffer in the pH range of 8.5–9.5, at pH intervals of 0.5. H1 was only marginally soluble below pH 7. The ionic strength of both the CP buffer and the glycine buffer was maintained constant at 0.15 (isotonicity) by the addition of appropriate amounts of NaCl. The dialysate was spun for 5 min at 17,000*g* at 5°C in a Sorvall Legend Micro 17R Centrifuge (ThermoScientific, Waltham, Massachusetts). The concentration of soluble protein was determined by UV spectroscopy using an Agilent 8453 UV–visible absorbance spectrophotometer (Agilent Technologies, Santa Clara, California) and measured in a quartz cuvette with a path length of 1 cm. Extinction coefficients of 2.05 and 2.27 mL/mg cm were calculated based on the sequence of H56 and H1, respectively (www.scripps.edu/~cdputnam/protcalc.html). It should be noted that the experimental conditions used in this study deviated from the drug product storage conditions.

### Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Unstressed recombinant proteins (4 μg) were run under nonreducing and reducing conditions on a precast NuPAGE® Bis–Tris 4%–12% gel using MES running buffer and 10 μL SeeBlue® Plus2 marker (Invitrogen, Carlsbad, California). The gel was stained with Coomassie blue (Bio-Rad, Berkeley, California).

### Far-UV CD Spectroscopy

Far-UV CD measurements were performed using a Chirascan CD spectropolarimeter (Applied Photophysics, Leatherhead, UK). The protein samples were prepared at a concentration of 0.2 mg/mL. Sealed quartz cuvettes with a 0.1 cm path length were used for all measurements. The spectra were recorded from 260 to 190 nm with a step size of 1 nm, a bandwidth of 1 nm, and an integration time of 0.5 s per point. To study thermal transitions, CD scans were obtained every 1.25°C over the temperature range of 10°C–87.5°C with an equilibration time of 30 s at each temperature. All spectra were background corrected and converted into molar elipticity.

### Intrinsic Tryptophan Fluorescence Spectroscopy and Light Scattering

Intrinsic fluorescence spectra were acquired with a Photon Technology International spectrofluorometer (Lawrenceville, New Jersey) equipped with a turreted four-position Peltier temperature controller. An excitation wavelength of 295 nm [>95% Trp emission] was used and the emission spectra were collected from 305 to 405 nm (slit widths of 2 nm) with a 1 nm/s data collection rate and 1 s integration time. The light scattering at 295 nm was collected simultaneously with the fluorescence emission employing a second photomultiplier in a 180° angle to the emission detector (slit width of 0.25 nm). Both fluorescence and light scattering were collected as a function of temperature between 10°C and 87.5°C with 2.5°C intervals and an equilibration time of 3 min at each temperature. All spectra were background corrected by subtracting the corresponding buffer spectrum. Fluorescence emission peak positions and intensities were determined by a center of spectral mass method (MSM) using Origin software (OriginLab, Northampton, Massachusetts). The center of spectral mass was determined as the median of the area under the curve (or spectra). This resulted in a 9–10 nm red shift of the peak position compared with the