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A systematic approach to stabilizing EBA-175 RII-NG for use as a malaria vaccine

Laura J. Peek, Duane T. Brandau, LaToya S. Jones [1], Sangeeta B. Joshi, C. Russell Middaugh *

Department of Pharmaceutical Chemistry, University of Kansas, 2095 Constant Avenue, Lawrence, KS 66047, USA

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Abstract

Region II of the erythrocyte-binding antigen (EBA-175 RII) has been identified as a promising target for a malaria vaccine. A systematic approach to identify optimal preformulation conditions of a non-glycosylated (NG) antigen, EBA-175 RII-NG, has been developed. This approach consists of development of an empirical temperature/pH phase diagram, high throughput stabilizer screening and aluminum salt adjuvant adsorption studies. Using these physical methods, we developed a stable formulation for EBA-175 RII-NG at pH 6.0 with sucrose and Brij® 35 as stabilizers and Adju-Phos® as an adjuvant. This approach should be generally applicable to guiding the development of stable vaccine formulations.

Keywords: Malaria; Stability; Protein

1. Introduction

More than 3 billion people are threatened each year by malaria, a parasite that kills over 1 million people annually [1]. For decades, the challenge of developing a malaria vaccine has been a primary focus in many research laboratories throughout the world. Many of the problems are due in part to the complexity of the life cycle of parasites that cause human malaria, most commonly *Plasmodium vivax* and *Plasmodium falciparum* [2,3]. *P. falciparum* has been the focus of many of these vaccines since this species is responsible for the majority of malaria infections and deaths seen worldwide [4,5]. Potential malaria vaccine antigens have been identified in an attempt to inhibit hepatocyte invasion by sporozoites, erythrocyte invasion by merozoites, as well as various other stages of growth and development throughout the parasite's life cycle [2,6].

A 175 kDa antigen was found to be involved in facilitating the invasion of erythrocytes by merozoites in 1985 [7]. This finding suggested that a vaccine antigen capable of blocking the organism's red blood cell attack might be a promising vaccine candidate. Certain regions in the gene of this antigen, specifically Region II (RII), are conserved among strains of *P. falciparum* and are responsible for binding of the organism to erythrocytes [8–10]. For these reasons, a non-glycosylated (NG) erythrocyte-binding antigen, EBA-175 RII-NG, is being pursued as a vaccine candidate.

A major problem in the development of any vaccine based on a recombinant protein is the creation of a stable, effectively adjuvanted formulation that will permit the vaccine to be stored and delivered anywhere in the world, even under adverse environmental conditions. We have, therefore, developed a systematic approach to guide the identification of optimal stabilizing conditions for EBA-175 RII-NG for use in a liquid injectable formulation. This approach involves the use of high-resolution second derivative absorbance spectroscopy, circular dichroism (CD), and both intrinsic and extrinsic fluorescence spectroscopies to monitor structural changes of the protein while undergoing thermal stress. Data

* Corresponding author. Tel.: +1 785 864 5813; fax: +1 785 864 5814.
E-mail address: middaugh@ku.edu (C.R. Middaugh).
[1] Present address: Department of Pharmaceutical Sciences, University of Colorado, 4200 E. 9th Avenue, C238, Denver, CO 80262, USA.

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from these physical techniques are combined to develop empirical phase diagrams, which define regions of similar physical states of the protein across a variety of pH and temperature conditions [11–15]. Based on the phase diagram, conditions for a high throughput screening of a library of generally regarded as safe (GRAS) excipients are selected. In this case, a turbidity assay was used for this purpose, and the ability of each excipient to inhibit protein aggregation was evaluated. Several excipients exhibiting significant inhibition of aggregation are then selected, and their effect on the conformational stability of the protein is evaluated using CD or fluorescence spectroscopies, or both. Conformational stabilizers are then analyzed in various combinations to determine if additional stabilization can be attained. It is critical that optimal stability is achieved so that the activity of the vaccine can be maintained even when a cold chain is not available [16] since malaria presents an especially difficult problem in tropical regions. Once the antigen's stability is optimized, adsorption isotherms are constructed to determine the amount of antigen that can be adsorbed to an aluminum salt adjuvant over a range of conditions.

2. Materials and methods

2.1. Materials

Citrate–phosphate (25 mM) buffers (pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0) containing 100 mM NaCl were prepared using citric acid monohydrate (Fisher, Pittsburgh, PA) and sodium phosphate dibasic, anhydrous (Sigma, St. Louis, MO). Sodium phosphate (10 mM) buffers (pH 6.0 and 7.2) were prepared using sodium phosphate dibasic, anhydrous, and sodium phosphate monobasic, monohydrate (Sigma, St. Louis, MO) and contained 150 mM NaCl. All excipients were purchased from Sigma (St. Louis, MO), except for sucrose octasulfate and Pluronic F68, which were purchased from Toronto Research Chemicals (Ontario, Canada) and Spectrum Chemical & Laboratory Products, Inc. (Gardena, CA), respectively. Sodium chloride was purchased from J.T. Baker (Phillipsburg, NJ).

Purified EBA-175 RII-NG was produced by Cambrex BioScience, Baltimore (Baltimore, MD) in collaboration with Protein Potential (Rockville, MD) and provided by Science Applications International Corporation under contract with the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The protein produced a single band on SDS-PAGE at approximately 2 mg/mL in 6 mM phosphate buffer, pH 7.4, containing 5% sucrose and 154 mM NaCl. Sucrose was used to inhibit dimerization detectable by size exclusion chromatography (SEC) upon thawing after freezing at −20 °C. This dimerization was not observed upon freezing at −70 °C (Protein Potential, personal communication). Protein concentration was determined by UV absorbance at 280 nm (A_{280}), using an extinction coefficient of 1.8 mL/mg cm, which was calculated using the Protein Calculator [17]. This tool employs Gill and von Hippel's method [18] to calculate the extinction coefficient from the number of tryptophan (Trp), tyrosine and cysteine residues in the protein.

Most protein dialyses were performed at 4 °C using Slide-A-Lyzer® Dialysis Cassettes, 10,000 MWCO (Pierce, Rockford, IL). In some cases, Spectra/Por® Cellulose Ester Sterile DispoDialyzers (2000 MWCO, 2 mL sample volume) (Spectrum Laboratories, Inc.; Rancho Dominguez, CA) were employed.

2.2. Biophysical characterization of EBA-175 RII-NG

The biophysical properties of EBA-175 RII-NG were evaluated at six pH conditions from pH 3–8.0 and over the temperature range of 10–85 or 90 °C, depending on the technique. In addition, the studies compared the properties of the antigen in the presence and absence of 5% sucrose, which was previously shown by size exclusion chromatography at Cambrex BioScience to prevent dimerization at −20 °C [19].

2.2.1. High-resolution absorbance spectroscopy

High-resolution absorbance spectra were obtained employing an Agilent 8453 UV–visible diode array spectrophotometer equipped with a Peltier temperature controller (Palo Alto, CA). Spectra were collected every 2.5 °C from 10 to 90 °C with a 5 min equilibration at each temperature. An integration time of 25 s was used to obtain spectra with a high degree of precision. The protein concentration used for each experiment was approximately 0.11 mg/mL. Each sample was evaluated in duplicate and analyzed from 200 to 400 nm. Turbidity traces were also generated for each sample by plotting the optical density at 360 nm (OD_{360}) as a function of temperature to monitor protein aggregation.

Second derivatives of the absorbance spectra were obtained by ChemStation™ software (Agilent) using a nine point data filter and the Savitsky-Golay smoothing function to fit data to a third order polynomial. The spectra were smoothed using 99 interpolated points between each data point, which resulted in highly resolved spectra on the order of ±0.01 nm [20]. Peak positions were selected by utilizing the Pick Peaks Tool in Microcal Origin™ 6.0. A width of 1.00 and a height of 10^{-4} were the dimensions of the box used to locate the negative peaks in each spectrum; a minimum height value was set to 5.00.

2.2.1.1. Empirical phase diagrams using second derivative absorbance spectroscopy data. Second derivative absorbance spectroscopy peak position data were used to generate empirical phase diagrams. These multi-colored plots display changes in the physical state of the protein as a function of temperature and pH. All calculations for construction of the phase diagrams were performed using Mathematica (Wolfram Research, Champaign, IL). The multi-component vector approach uses *n*-dimensional vectors composed of data at each combination of pH and temperature to generate