KIMIKA Volume 20, Number 1/2, pp. 13–16 (2004)

ISSN 0115–2130

Binding of congo red and crystal violet with β-2-microglobulin amyloid fibril

Mara Teresa T. Panlilio[1], Noel S. Quiming[3], Rex Vergel[1,2], and James A. Villanueva[1,2]*

[1]Institute of Chemistry & [2]Natural Sciences Research Institute
University of the Philippines
Diliman, Quezon City
Metro Manila 1101, PHILIPPINES

[3]Department of Physical Sciences & Mathematics
University of the Philippines
Manila, PHILIPPINES

Dialysis-Related Amyloidosis (DRA) is a serious complication for patients undergoing long-term hemodialysis and severe renal failure. Its clinical manifestation (i.e., Carpal Tunnel Syndrome) may be associated mainly with fibril deposits of β-2-microglobulin (β2M) in the joints, kidney, and bone. Previous studies have been made to show the binding of dyes such as congo red (CR) and Thioflavin T to β2M fibrils. Since staining methods for the demonstration of ß2M fibrils are limited, other dyes such as crystal violet (CV) may be developed as stains to elucidate amyloid fibrils. UV-Vis spectroscopy was used to demonstrate the shift in the maximum absorption wavelengths, which indicates binding, of the β2M seeded solutions examined. The use of UV-Vis spectroscopy as a technique for amyloid fibril demonstration serves as an advantage due to the instrument's availability in most laboratories. β2M solutions tested with CV demonstrates a shift (to shorter wavelengths) in the maximum absorption wavelengths. Thus, the development of these dyes as methods for amyloid elucidation will not only increase the variety of staining methods, but also will help design new therapeutic strategies to combat amyloidosis.

Keywords: β-2-microglobulin; amyloid; crystal violet; congo red

INTRODUCTION

Specific aggregation of several proteins into long, non-covalent, and extra-cellularly deposited amyloid fibrils leads to the pathological disorder known as amyloidosis. This disease is carried out by the deposition of soluble proteins into insoluble fibrils having β-pleated sheet structures [1]. It is also further characterized through clinical syndromes such as Alzheimer's disease, senile dementia, hemodialysis, and other manifestations of the cerebral amyloid.

β-2-microglobulin (β2M), the light chain of the type I major histocompatibility complex, builds up amyloid plaques in patients undergoing long-term hemodialysis. This expression brings about localized β2M amyloidosis, which can progressively replace the parenchyma cells of the vital organs, thus the organ losing its function, since the protein cannot pass through the conventional dialysis membranes. Since the mechanism of formation of β2M fibrils is still unknown, several studies and methodologies have been made to clarify β2M fibrillogenesis. With this, staining methods have been developed to elucidate the β2M amyloid. Congo red (CR) and Thioflavin T are the most universally used dyes for this purpose. Previous studies on amyloid fibril formation have always referred to these methods [2].

Since the two aforementioned dyes are considered to be used in main staining methods for the identification of the amyloid, other dyes exhibiting similar properties to CR and Thioflavin T are under study, as possible alternatives to these dyes. Crystal

**To whom correspondence should be addressed.*

violet (CV) is proposed to be tested for its specificity and sensitivity to amyloid demonstration. Thus, it is the aim of this study to show the staining action of CV along with its binding, under controlled conditions, to β2M. This study is in line with the aim of increasing the variety of staining methods for protein fibril formation. The results may also facilitate proper targeting and design of fibrillogenesis inhibitors necessary in the prevention of amyloidosis.

MATERIALS AND METHODS

Sample solution preparation for dye-binding experiments. Seeded and unseeded sample solutions are prepared as described by Ohhashi et al. [3]. An unseeded solution of 200 μM monomeric β2M in 50 mM citrate buffer (at pH 2.5) and 100 mM KCl at 4°C were prepared. To prepare the seeded solution of β2M, 10 μL of prepared fibrils were added to 1mL of 25 μM monomeric β2M. Resulting solutions were then used for the CV binding studies.

Preparation of fibril solution. Fibrils were prepared by addition of 50 μL of concentrated β2M seeds, which were sonicated, to 1 mL of 100 μM monomeric β2M. Ten (10) μL of 0.1% (w/v) NaN_3 may be added to the solution as preservative. The resulting solution was incubated at 37°C for eight days. Fibril formation may be followed and observed by using the Thioflavin T Assay.

Congo red spectral shift assay. The CR spectral shift assay was performed using simple spectrophotometric methods. 100 μM of CR (Direct Red 28 from Ajax Laboratory Chemicals) in a solution of 90% phosphate-buffered saline (PBS: 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl) at pH 7.4 and 10% (v/v) ethanol was prepared. The stock solution was filtered using Whatman 0.2 μm nylon membrane filters to remove micelles of CR. The concentration of the stock solution was determined by constructing a calibration curve with diluted standards ranging from 10–15 μM CR in 1 mM potassium phosphate (pH 7.0) and 40% ethanol. The absorbances of the solutions were read at 505 nm ($\varepsilon = 5.93 \times 10^4\ M^{-1}\ cm^{-1}$) using a Shimadzu UV-3101 UV-Vis-NIR scanning spectrophotometer.

Test solutions containing CR and β2M were prepared by keeping the 1 CR: 5 β2M concentration ratio. Varying amounts of CR were added in the cuvette to give 4, 8, 12, 16, and 20 μM of CR. Corresponding amounts of β2M were added to the cuvette to follow the aforementioned concentration ratio. The test solutions were then scanned for its maximum absorption wavelengths. Solutions (of the same CR concentration) containing seeded β2M, instead of monomeric β2M, were also tested. In addition to these, control samples containing CR alone were also subjected to the assay.

Crystal violet assay. This assay is presented as described by Prophet et al. [4] and Lieb E [5]. The CV (LabChem) stock solution is prepared by mixing 7.0g of CV and 50.0 mL of 95% ethanol. A CV working solution of 200 μM in concentrated HCl /ater was then prepared. At CV concentrations of 2, 4, 6, 8, and 10 μM, test solutions (addition of 10 μM β2M Monomer and seeded solutions) and control samples (CV alone) were scanned using the spectrophotomer for the maximum absorption wavelengths. Fibril extension studies were also performed by observing the changes in absorbance with time of a solution of 6 μM of CV with 7.5 μM β2M seeded solution.

RESULTS AND DISCUSSION

β-2-microglobulin fibril formation. β2M is transported by the serum to the proximal tubule of the kidney, where it is degraded and excreted [2]. It is by an unknown mechanism that as a consequence of this transport, β2M forms amyloid fibrils that are predominantly deposited in the musculoskeletal system, but one proposed mechanism is via nucleation-polymerization [1]. These fibrils may be formed *in-vitro* by incubation of recombinant wild-type β2M at low pH and high ionic strength. Acidification at below pH 5 ensures formation of fibrils. At pH 2.5 and at low ionic strength (50 mM), the protein adapts a state of long and straight fibrils. At pH 1.5–5.0 and high ionic strength (400 mM), fibrils formed are of curved morphology [1]. In addition to these, protofibrillar structures are said to be formed initially before the assembly into β2M fibrils. These structures are formed at pH 3.6.

The conditions applied in the experiments follow the desired conditions for β2M fibril formation. Solid β2M was dissolved in citrate buffer of pH 2.5 and 100 mM KCl. This monomeric solution of β2M would form fibrils upon incubation at 37°C, and may be detected using Thioflavin T. To accelerate fibril formation, the concentration of the β2M solution was increased from 25 μM to 100 μM. The monomeric solution was seeded with concentrated and sonicated β2M fibrils. On the other hand, at neutral pH, β2M is stable, yet fibril formation does not proceed even after long periods of incubation. Thus, a reduced pH environment is necessary for the formation of β2M fibrils *in-vitro*.

Congo red binding. Hydrophobic interactions are said to have been the possible mechanism of the CR binding to the β2M fibril. Another study suggests that the CR molecule consists of two identical halves [6]. The considered binding site in the β2M fibrils is the interface between the monomers of antiparallel β-sheets. This antiparallel condition of the amyloid fibrils allows the binding of similar chemical environment to both halves of the CR molecule.

CR also induces a green birefringence in polarized light. According to Klunk, et al. [7], the mechanism of the stereochemical binding of amyloid proteins involves the β-pleated structure orienting the CR molecule to conformational alignment, and eventually, two positively-charged amino acids in the primary structure are attracted to the negatively-charged sulfonic groups found in the CR molecule [6]. This is deduced

from the study that the distance between every 5th β-pleated sheet (4 × 4.7 Å) is very close to the distance between the two sulfonic groups (19.3 Å) in the CR molecule [7]. Thus, parallel binding of CR with the fibril connects two β-pleated sheets. This then allows the special optical appearance, the apple green birefringence, under polarized light.

In this work, the binding of the CR molecule to the β2M fibril is verified through the spectral shifts observed (Fig. 1). A red shift, or a shift to longer wavelengths is observed when a solution of β2M fibril and CR is scanned in the UV-Vis region. A solution of β2M monomer and CR does exhibit a shift in its maximum absorption wavelength when compared to the control

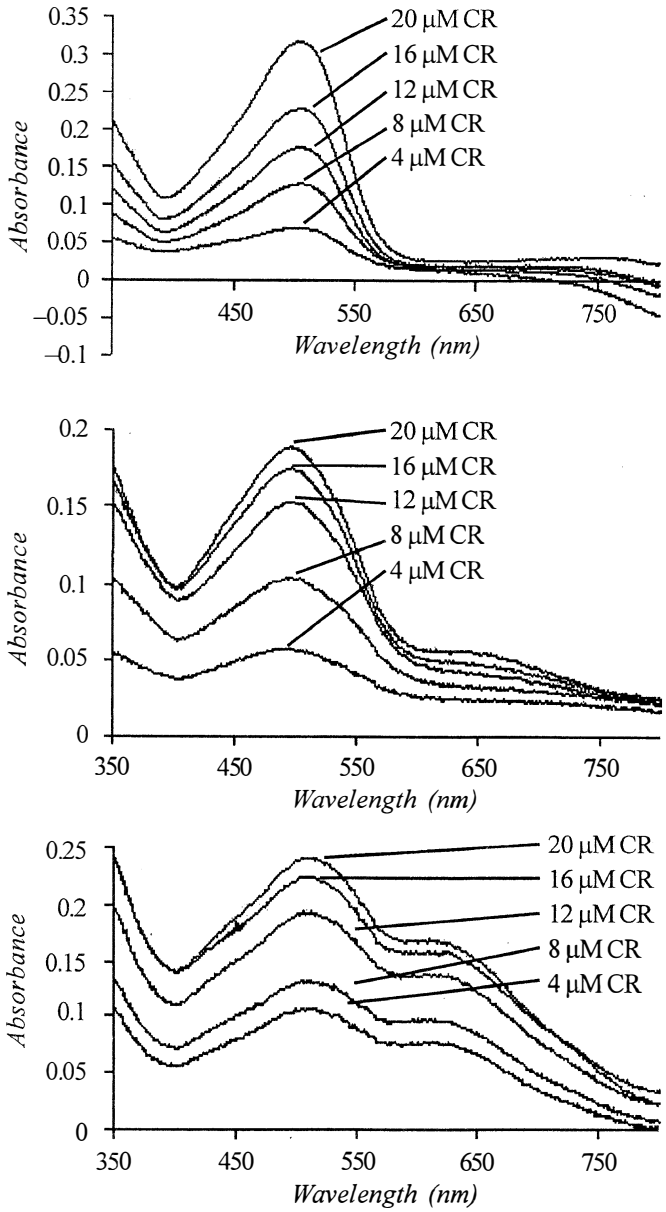

Fig. 1. Absorption spectra of congo red (a) *alone, with* (b) *b2M Monomer, and with* (c) *β2M fibril. Reaction mixtures of Congo red (CR) and β2M solution with fibril induced a spectral shift to longer wavelengths as compared to mixtures of CR and β2M monomer. In addition to this, absorbance at a preset wavelength (taken as the average maximum absorption wavelength of each solution) increases as the concentration of congo red is increased.*

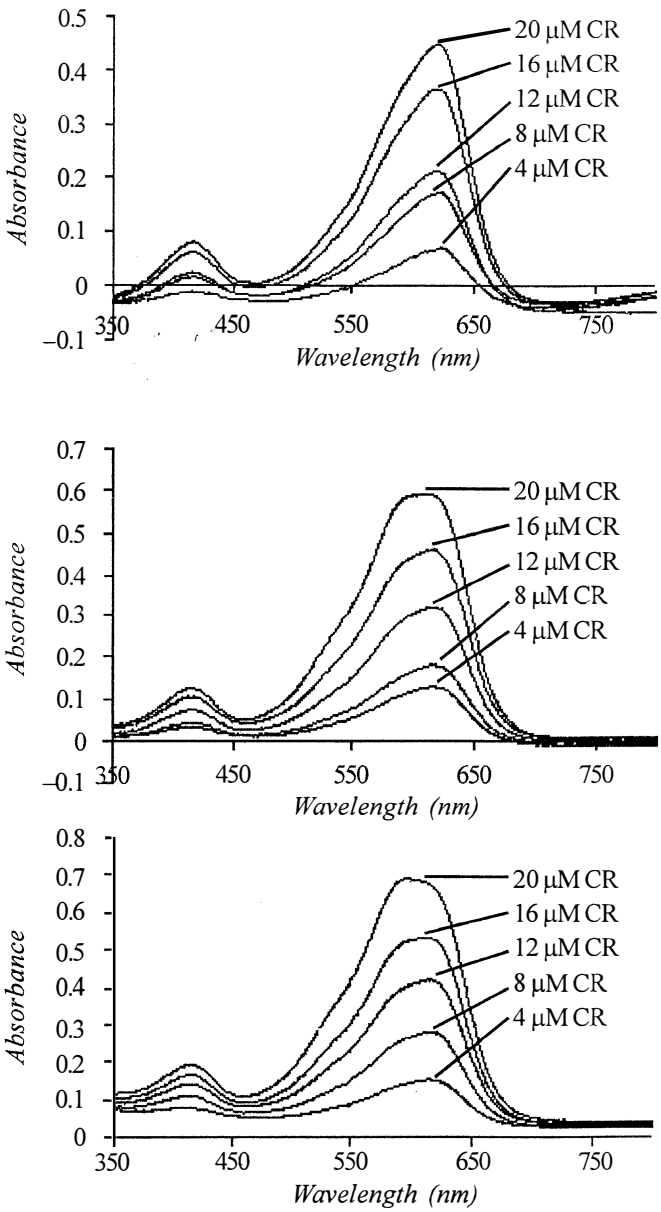

Fig. 2. Absorption spectra of crystal violet (a) *alone, with* (b) *β2M Monomer, and with* (c) *β2M fibril. A shift to shorter wavelengths was observed between the control samples and the solutions of β2M fibrils and monomer and crystal violet (CV). In contrast to the other binding assays done, there was no spectral shift observed between the solutions of β2M fibrils and CV, and β2M monomer and CV.*

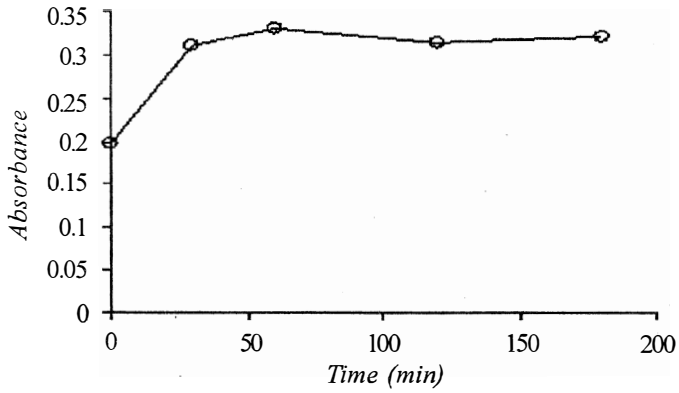

Fig. 3. Fibril extension studies using crystal violet. The time profile study shown above illustrates that as the β2M fibrils were incubated through time, the absorbance measurements increase. A solution of 6 μM of CV with 7.5 μM β2M seeded solution incubated at 37°C was used in this experiment.

sample solutions (CR alone). Also, it was observed that as the concentration of the dye is increased, the absorbance of the solution increases.

Crystal violet binding. The mechanism by which CV binds to the β2M fibril was not established in this work. Based on previous studies and research, it was known that CV demonstrates the amyloid via metachromasy. In histology and tissue staining, this would mean that the color of the positive result for amyloid would elicit a different color with that of the dye itself. Reaction solutions were light blue, while the dye was strikingly purple. This, though, is not enough proof that metachromatic demonstration was achieved.

In other previous works, metachromasy is exhibited by a blue shift, or a shift to shorter wavelengths. This was observed in the solutions of β2M fibrils and CV, as compared to the control samples (CV alone) (Fig. 2).

Fibril extension studies were also done, wherein it was seen that the absorbance measurements increase as the time of incubation of the fibril increases (Fig. 3). Thus, further studies on the CV binding must be done to investigate the metachromatic demonstration of the amyloid fibril and confirm its binding.

ACKNOWLEDGMENT

The authors would like to thank the Analytical Services Laboratory of the Institute of Chemistry in UP Diliman for the use the instruments and Dr. Yuji Goto of the Institute for Protein Research, Osaka University, Japan.

REFERENCES

1. Astbury Centre for Structural Molecular Biology. Annual Report 2001. Radford, S., Brittain, A., Gidalevitz, T., Jones. S., McParland, V., Myers, S., Smith, D., Stilling, C. Ashcroft, A., Thomson, N., and Smith, A. United Kingdom, 2002.

2. Jones, S., Manning, J., Kad, N. M., and Radford, S. E. *J. Mol. Biol.* 325, 249 (2003).

3. Ohhashi, Y., Hagihara, Y., Kozhukh, G., Hoshino, M., Hasegawa, K., Yamaguchi, I., Naiki, H., and Goto, Y. *J. Biochem.* 131, 42 (2002).

4. Prophet, E.B., Mills B., Arrington J.B. and Sobin L.H. (Eds.) *American Registry of Pathology*. (Armed Forces Institute of Pathology. Laboratory Methods in Histochemistry; Washington, D.C., 1992).

5. Lieb E. *Am J Clinical Pathology.* 17, 413 (1947).

6. Haviv, H. S., Shalit, M., Cohen, R., Pizov, G., and Muszkat, M. *Clinical Nephrology.* 54, E4 (2000).

7. Klunk, W. E., Pettegrew, J. W., et al. *J. Histochem. Cytochem.* 37, 1272 (1989). Abe, H. and Nakanishi, H. *Analytical Sciences.* 19, 171 (2003).