Hydrophobic profiling of heated and unheated Leghemoglobin, Myoglobin, Cytochrome c and Hemoglobin proteins in differential pH conditions

Rittik Kumar Ghosh[1],Turban Kar [2], Pijush Basak[*3] and Maitree Bhattachayya[*4]

[1,2,3,4] Department of Biochemistry, University of Calcutta, 35, Ballygunge Circular Road, Kolkata - 700019, West Bengal, India .
[3,4] Jagadis Bose National Science Talent Search , 1300, Rajdanga Main Road, Sector C, East Kolkata Township, Kolkata-700107, West Bengal, India

Abstract- Surface hydrophobicity of leghemoglobin (Lb), myoglobin (Mb) ,cytochrome c (Cyt C) and hemoglobin (Hb) under heated (80 °C for 30 min.) and unheated conditions and at varying pH values (3.0, 5.0, 7.0, and 9.0) were measured using ANS, CPA, and PRODAN. Hydrophobicity was lower at pH 9.0, 5.0 and 7.0 for all proteins measured by PRODAN, for ANS and for CPA respectively. Lb shows maximum hydrophobic nature with ANS and CPA may and less with PRODAN compared to other heme proteins, indicating that Lb favor less ionic interaction. These results suggest that the presence of a permanent charge of fluorescent probes can affect protein hydrophobicity values measured under various pH conditions for Lb.

Index Terms- Heme proteins, pH, Surface hydrophobicity, external fluorescence probe.

I. INTRODUCTION

Characteristics of amino acids can be relatively studied by protein hydrophobic measurements. They are one of the most important non covalent interactions in nature [1]. Amino acids vary in the polarity of side chains which are primary characteristics of the structure of the protein, useful in the identification of protein interactions. The distribution of hydrophobic and hydrophilic amino acids helps in the classification of tertiary structure, which in turn influences the quaternary structure.

Hydrophobicity is heterogeneously distributed in protein surface. A parameter called local hydrophobicity, defined as the average surface hydrophobicity of an interaction zone with probe is used to determine hydrophobic interaction, which essential establishes a protein's stability [2]. Binding of extrinsic fluoro probes ANS (anilino-1-naphthalene sulphonic acid), CPA (cis-parinaric acid) and PRODAN (6-propionyl 1, 2-dimethylamino naphthalene) to the accessible surface of a protein is used to measure the surface hydrophobicity of a protein [3-5]. These probes are extensively used to quantify protein hydrophobicity, to monitor conformational changes in biological macromolecules and to study protein binding sites [6-8]. Anionic Probes have both electrostatic and hydrophobic interactions with the proteins. It is very difficult to measure the accurate protein surface hydrophobicity. To overcome this problem neutral fluorescent probe PRODAN was used [9]. ANS and PRODAN are sensitive to the polarity of the environment in biological materials, both ANS and CPA are considered to be anionic probes. Under acidic and alkaline pH, a contribution of charged interactions on the measurement of surface hydrophobicity using the anionic fluorescent probes may be expected. PRODAN, on the other hand, is a solvent-sensitive probe and has no charge. This will eliminate possible electrostatic interaction contributions in the measurement of protein hydrophobicity [10].

The objectives of this study were to establish a fluorescent probe method using an uncharged probe (PRODAN), prepared as a methanol stock solution, to compare the values of protein surface hydrophobicity to that of aliphatic (CPA) and aromatic (ANS) anionic probes. Cations and anions do not appear to have equal access to the same binding sites. The use of neutral or uncharged probes may circumvent this problem. Surface hydrophobicity of four protein samples at various pH values were measured using these three probes.

II. MATERIALS AND METHODS

2.1 Protein sample preparation

Leghemoglobin isolated and purified from ground nut *Arachis hypogea*, according to Basak et al [11] . Myoglobin (Sigma, USA), Hemoglobin isolated according to Mondal et al [12] and Cytochrome C (Sigma, USA) was used for our purpose. Fluorescent probes, ANS, CPA, and PRODAN were obtained from Sigma (USA). Buffers of pH 3.0, 5.0, 7.0, 9.0 were prepared according to the method of Dawson et al [13].

2.2 Hydrophobicity Determination

Protein surface hydrophobicity (S0) was determined for using ANS, PRODAN and CPA probes [12] . Stock solutions of 5mM ANS, 2.4mM PRODAN, and 3mM CPA were prepared in 0.1M phosphate buffer of pH 7.4, methanol and ethanol respectively. Concentrations of the ANS, PRODAN and CPA stock solutions were determined spectrophotometrically at 350, 360 and 303nm, respectively, using molar absorption coefficients of ANS-4.95 x 103 M-1cm-1 [14] , CPA-7.6 x104M-1cm-1 [6], and PRODAN-1.8 x 104 M-1 cm-1 [4] respectively. To prevent evaporation, CPA and PRODAN stocks were stored into screw-capped vials, sealed with parafilm. ANS stock solution was stored in a screw-capped container at room temperature but PRODAN and CPA stocks were stored in -100C and throughout the experiment time period they were always stored in ice. All fluorescence measurements were made with Hitachi F7000 Spectrofluorometer. To measure protein surface hydrophobicity

using PRODAN, the excitation/emission slits and wavelengths were set at 5 nm/5nm and 365 nm/465 nm, respectively. 3μM protein, was mixed with probes, for a period of 15 min in the dark, Then Relative fluorescence intensity (RFI) of each solution was measured including buffer blank (buffer plus probe) and then from the lowest to the highest protein concentration. RFI values of buffer and protein dilution blanks (no PRODAN) were also measured. To obtain net RFI, the RFI of each protein dilution blank was subtracted from that of corresponding protein solution with PRODAN. To measure hydrophobicity using ANS excitation/emission slits and wavelengths were set at 5 nm/5 nm and 390 nm/470 nm, respectively and for CPA excitation/emission slits and wavelengths were set at 5 nm/5 nm and 325 nm/420 nm, respectively. The initial slope (S0) of the net RFI versus protein concentration plot was calculated with Microsoft Excel for Windows7 is used as an index of the protein surface hydrophobicity.

III. RESULTS AND DISCUSSION

The surface hydrophobicity values determined for four native and heated proteins and was assessed using uncharged [PRODAN] versus anionic aliphatic (CPA) and aromatic (ANS) probes. Surface hydrophobicities of Lb ,Mb, Cyt C and Hb under heated (80 °C for 30 min) and unheated conditions and at varying pH values (3.0, 5.0, 7.0, and 9.0) were measured using ANS, CPA, and PRODAN.

For all heme proteins the lowest hydrophobicity value using the ANS method was observed at pH 5.0 (see Fig 1). At pH5 the trend is Hb>Lb>Mb>Cyt C for unheated sample and for the heated sample the observed value is different, here S_0 of Lb, Cyt C, Mb are almost same and greater than Hb. At pH7,the S_0 of Lb>Cyt C>Hb>Mb for unheated sample and for heated sample the trend is slightly different, here the trend is Lb>Cyt C>Mb>Hb. At pH9, the hydrophobicity of four different proteins is in the order of Lb>Mb>Cyt C>Hb for heated sample and Lb>Cyt C>Mb>Hb for unheated sample.

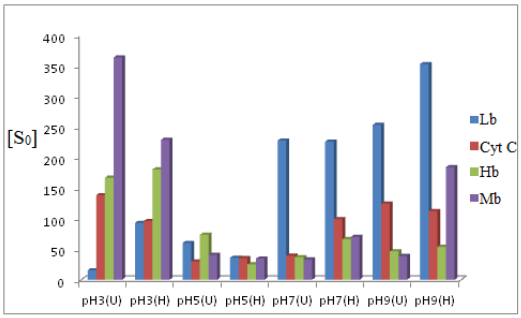

Figure 1: Hydrophobicity measurements of heated and unheated samples of Lb, Cyt C, Hb and Mb using ANS. S_0 denotes protein hydrophobicity values.

At pH3 in presence of CPA, the hydrophobicity order is Lb>Mb>Hb>CytC for unheated sample and Lb>Mb>Hb>Cyt C for heated sample (see Fig 2). At pH5, the trend is Lb>Mb>Hb>Cyt C for unheated sample and Mb>Lb>Hb>Cyt C for the heated sample. At pH9, the hydrophobicity of 4 different proteins is in the order of Lb>Mb>Hb>Cyt C for heated sample and Lb>Cyt C>Mb>Hb for unheated sample. For all four proteins the lowest hydrophobicity value using the CPA method was observed at pH 7.0 At pH7, the hydrophobicity of Hb>Mb>Cyt C>Lb for unheated sample and for heated sample the trend is slightly different, here the trend is Mb>Lb>Hb>Cyt C.

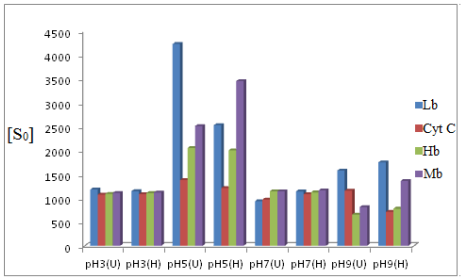

Figure 2: Hydrophobicity measurements of heated and unheated samples of Lb, Cyt C, Hb and Mb using CPA. S_0 denotes protein hydrophobicity values.

At pH3, the hydrophobicity of Mb>Lb>Cyt C>Hb for unheated sample and for heated sample the order is Hb>Lb>Mb>Cyt C in presence of PRODAN (see Fig 3). At pH5, the trend is Cyt C>Hb>Mb>Lbfor unheated sample and for the heated sample the trend is Cyt C>Mb>Hb>Lb. At pH7, the hydrophobicity of Hb>Cyt C>Mb>Lb for unheated sample and for heated sample the trend is slightly different, here the trend is Lb>Mb>Hb>Cyt C. For all four proteins the lowest hydrophobicity value using the PRODAN method was noted at pH 9.0. At pH9, the hydrophobicity of proteins are Mb>Lb>Cyt C>Hb for heated sample and Mb>Cyt C>Lb>Hb for unheated sample.

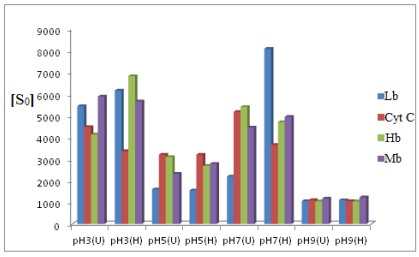

Figure 3: Hydrophobicity measurements of heated and unheated samples of Lb, Cyt C, Hb and Mb using PRODAN. S_0 denotes protein hydrophobicity values.

Fluorescence probes have low quantum yield in aqueous solution. Upon binding of the probes to accessible hydrophobic regions of proteins, the observed fluorescence, increment is used as a measure of protein hydrophobicity. The influence of fluorescent probe on the surface hydrophobicity values for all heme proteins the lowest hydrophobicity value using the ANS method was observed at pH 5.0.There were no significant differences in the intensity obtained from unheated and heated heme proteins. The index of ANS binding to heme proteins at pH 5.0 suggests that utilization of hydrophobic domains formed after denaturation of heme proteins is low compared to other pH induced states indicating significant or high level of utilization of hydrophobic domains formed at pH 3.0, 5.0 and 9.0.

Simultaneously highly hydrophobic nature was observed in native state at all pH, compared to denatured states. Less hydrophobicity is achieved during denaturation of protein, which may destroy the conformation and hence leading to less hydrophobic interactions contributing a barrier for ANS in the solvent during interaction.

But the lowest hydrophobicity value using the CPA and PRODAN method was observed at pH 7.0 and pH 9.0 respectively for all heme proteins. Both PRODAN and ANS showed similar trend possibly due to similar hydrophobic interactions between ANS- or PRODAN and a protein. Both ANS and PRODAN contain a naphthyl moiety, and probably bind to a common region of the protein molecule. In case of CPA different results were observed during denatured state compared to ANS and PRODAN. CPA interaction with heme protein exhibited highly hydrophobic behavior at pH 5.0, 7.0 and 9.0 indicating that sulfonate and carboxylate groups may be formed at the pKa values of the groups present at the pH of the environment. Under these conditions of lower acidic to alkaline pH, a contribution of charged interactions on the measurement of surface hydrophobicity is favorable.

IV. CONCLUSION

It is expected that surface hydrophobicity should increase when the molecule unfolds while heating. Unfolding may be followed by protein aggregation through hydrophobic interactions or SH/SS inter change reactions. The intermolecular interactions may lead to decreases in surface hydrophobicity, primarily unfolding of proteins leading exposure of hydrophobic sites, and decreases in the exposure of hydrophobic sites and loss of solubility by forming aggregation . Lb shows maximum hydrophobic nature with ANS and CPA and less with PRODAN compared to other heme proteins indicating that the presence or absence of a permanent charge as well as the aromatic and aliphatic nature of fluorescent probes can affect protein hydrophobicity values measured under various pH conditions.

V. ACKNOWLEDGEMENTS

This work was supported by grant from UGC-UPE. We also acknowledge DST (FIST), World Bank, ICZMP, UGC-CAS and DBT-IPLS Government of India for providing the instrumental facility in the Department of Biochemistry, C.U.

REFERENCES

[1] BrianW.Mathews,"Hydrophobic interaction in proteins", *John Wiley & Sons, Ltd*, 2001.

[2] Pace CN, Fu H, Fryar KL, et al. "Contribution of hydrogen bonds to protein stability". *Protein Science*, 23(5), 2014, pp 652-661.

[3] M Cardamone and N K Puri, "Spectrofluorimetric assessment of the surface hydrophobicity of proteins." *Biochemical Journal*, 282, 1992, pp 589-593.

[4] Chakrabarti, A. "Fluorescence of spectrin-bound prodan." *Biochemical and biophysical research communications*, 226, 1996, pp 495-497.

[5] Haskard, C. A., Li-Chan, E. C. Y., "Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged(PRODAN) and anionic (ANS) fluorescent probes". *Journal of Agricultural and Food Chemistry*, 46, 1998, pp 2671-2677.

[6] Haughland, R. P., "Fluorescent phospholipids, fatty acids and sterols". *Handbook of Fluorescent Probes and Research Chemicals*; *Molecular Probes*, 1996, pp 299-300.

[7] Hudson, B. S., Harris, D. L., Ludescher, R. D., Ruggiero, A., Cooney-Freed, A., Cavalier, S. A., "Fluorescent probe studies of proteins and membranes", *Applications of Fluorescence in the Biomedical Science*, 1986, pp 159-160.

[8] Rettig W., "Kinetic studies on fluorescence probes using synchrotron radiation." *Fluorescence Spectroscopy*, *Wiley-Springer*, 1993, pp 31-32.

[9] Weber, G. Farris, F. J., "Synthesis and spectral properties of ahydrophobic fluorescent probe: 6-propionyl-2-(dimethylamino)naphthalene." *Biochemistry*, 1979, 18, pp 3075-3078.

[10] Hermetter, A., Prenner, E., Loidl, J., Kalb, E., Sommer, A.Paltauf, F., "Ether phospholipids in membranes: applications of phase and steady-state fluorometry." *Fluorescence Spectroscopy*, *Wiley-Springer*,1993, pp 149-150.

[11] Basak, P.,Bhattacharyya M.,"Intrinsic tryptophan fluorescence and related energy transfer in leghemoglobin isolated from *Arachis hypogea*." *Turkish Journal of Biochemistry*, 2013, 38, pp 49-56.

[12] Mondal, B., Chatterjee, D., Bhattacharyya M., "Structure-function alteration of hemoglobin in arsenicosis patients: a probable pathway to exert toxicity." *Journal of Applied Toxicology*, 2012, 32, pp 581-589.

[13] Kato, A., Nakai, S., "Hydropobicity determined by a fluorescentprobe method and its correlation with surface properties of proteins." *Biochimica Biophysica Acta*, 624, 1980, pp 13-20.

[14] Carolyn A., Haskard and Eunice C. Y. Li-Chan, "Hydrophobicity of Bovine Serum Albumin and Ovalbumin Determined Using Uncharged (PRODAN) and Anionic (ANS^-) Fluorescent Probes."*Journal of Agricultural and Food Chemistry*, 46, 1998, pp 2671–2677.

AUTHORS

First Author - Rittik Kumar Ghosh , Department of Biochemistry, University of Calcutta , Kolkata, West Bengal, India , Email:rittikghosh@ymail.com
Second Author – Turban Kar , Department of Biochemistry, University of Calcutta , Kolkata, West Bengal, India , Email:turbankar@gmail.com
First Corresponding Author - Pijush Basak , Jagadis Bose National Science Talent Search , Kolkata, West Bengal, India , Email:basakpijush@gmail.com
Second Corresponding Author - Maitree Bhattacharyya , Biochemistry, University of Calcutta , Kolkata, West Bengal, India , Email:bmaitree@gmail.com