COMPARATIVE STUDY OF CERTAIN FERRIC AND FERROUS DERIVATIVES OF HEMOGLOBIN LABELED WITH HPT

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Abstract. The purpose of this survey is to highlight certain aspects related to the structure-function relation that governs the existence of hemoglobin as an allosteric protein using absorption and fluorescence spectroscopy techniques, and a number of substances known as allosteric effectors of hemoglobin. This paper presents a method of indirect fluorescence labeling of hemoglobin and its derivatives with 8-hydroxy-1,3,6 pyrentrisulfonic acid trisodium salt (HPT), a functional analogue of 2,3,-biphosphoglycerate (BPG), possessing more favorable physical properties, considering that heme is a strong quencher of any fluorescent molecule that might be used for the direct labeling of hemoglobin. Fluorescence spectra were recorded for oxyhemoglobin labeled with HPT, methemoglobin labeled with HPT, cyanhemoglobin labeled with HPT and fluorhemoglobin labeled with HPT.

Key words: oxyhemoglobin, methemoglobin, cyanhemoglobin, fluorhemoglobin, HPT.

INTRODUCTION

HPT is a fluorescent analogue of BPG, which binds hemoglobin with a stoichiometry of 1 HPT/1 tetramer and with an affinity comparable to that of BPG. The same as BPG, HPT binds to deoxyHb with a higher affinity than to oxyHb [5]. Once bound, the fluorescence of HPT is quenched by the transfer of energy to heme and thus it can be used as a proof for the allosteric equilibrium: strong quenching indicates strong binding and thus a configuration that is mostly present in the T state [6]. In this paper we used these properties of HPT to investigate the conformational state (R-T equilibrium) of some of the ferric and ferrous derivatives of hemoglobin, according to pH values.

MATERIALS AND METHODS

An UV/VIS GBC Cintra 10e spectrophotometer and an Aminco-Bowman spectrofluorometer were employed for investigation of the absorption and fluorescence spectra.

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The hemoglobin was extracted from human blood using the classic method: we started from 4 ml of fresh human blood collected on anticoagulant (sodium citrate). We did 4 washings with an isotonic solution of NaCl 1% and 4 centrifugations at 5000 rotations/minute for 15 minutes. After each centrifugation we kept the sediment that contains red cells. Red cells lysis was done with distilled water at 4 °C for 30 minutes. The separation of hemoglobin was performed by centrifugation at 15000 rotations/minute for 40 minutes.

We prepared solutions of oxyhemoglobin, methemoglobin, cyanhemoglobin, fluorhemoglobin in Tris buffer, and solution of HPT, each of them at different pH values. From their absorption spectra we measured the concentrations of the solutions used in our experiment [7]. For the proper study of fluorescence, we prepared solutions of oxyHb+HPT, metHb+HPT, fluorHb+HPT, cyanHb+HTP.

RESULTS AND DISCUSSIONS

Studies performed on free HPT. Figure 1 shows the emission fluorescence spectra of HPT in Tris buffer solution between 450 – 600 nm. The unique band observed has a maximum intensity at 510 nm and its position is independent of pH value.

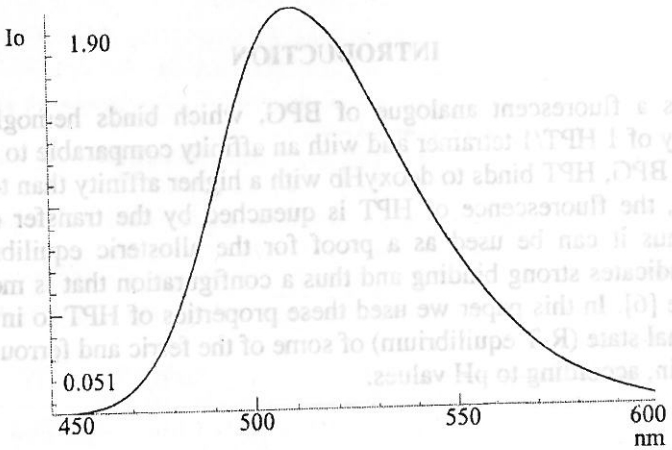

Fig. 1. – The static fluorescence spectrum of free HPT in Tris buffer solution (pH = 7).

The excitation wavelength used is 300 nm and is located in the nearness of the first isosbestic point with the property that extinction does not depend on pH (Fig. 2). There are three isosbestic points in the absorption spectrum of HPT located at 300 nm, 340 nm and 420 nm. The reason why we selected $\lambda_{excit} = 300$ nm is because the extinction value is lower at 340 nm and at 420 nm the hemoglobin absorption is more evident.

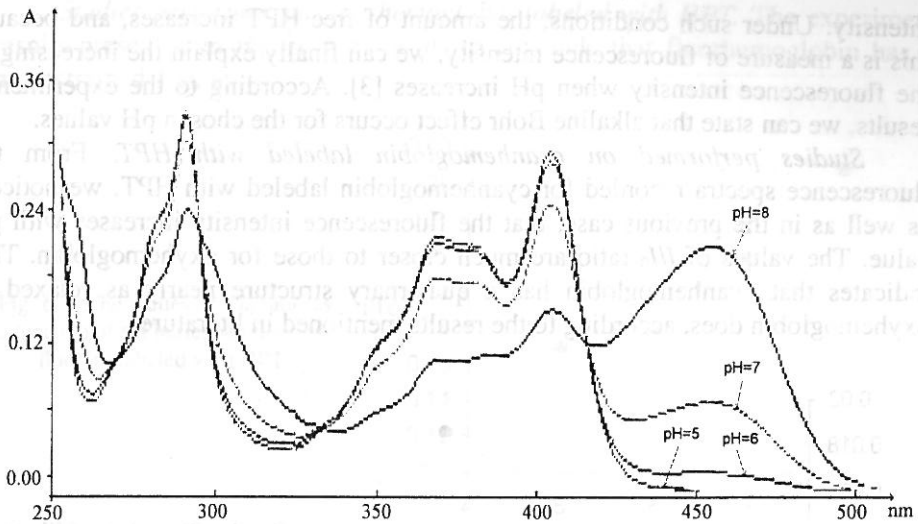

Fig. 2. – The absorption spectrum of free HPT in Tris buffer solution, at different pH values.

Studies performed on oxyhemoglobin labeled with HPT. For the experiment, we prepared four samples at different pH values. The fluorescence spectra recorded indicate that the position of maximum at 510 nm does not depend on pH value.

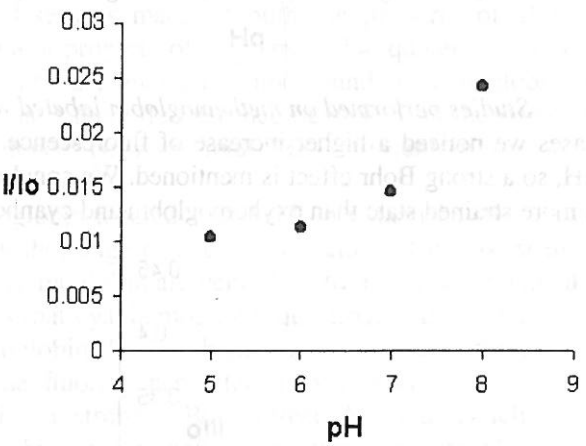

Fig. 3. – The values of I/I_0 ratio as a function of pH buffer solution for oxyHb labeled with HPT.

On the other hand, the maximum fluorescence intensity increases as pH increases. Knowing that the HPT fluorescence intensity practically presents a weak variation with pH, we performed a standardization of the fluorescence intensities (I) of the four samples at the values of the fluorescence intensity of free HPT (I_0).

As a result of increasing the affinity to oxygen, hemoglobin undergoes a light conformational relaxation that determines the decreasing of the HPT bound

intensity. Under such conditions, the amount of free HPT increases, and because this is a measure of fluorescence intensity, we can finally explain the increasing of the fluorescence intensity when pH increases [3]. According to the experimental results, we can state that alkaline Bohr effect occurs for the chosen pH values.

Studies performed on cyanhemoglobin labeled with HPT. From the fluorescence spectra recorded for cyanhemoglobin labeled with HPT, we noticed, as well as in the previous case, that the fluorescence intensity increases with pH value. The values of I/I_0 ratio are much closer to those for oxyhemoglobin. This indicates that cyanhemoglobin has a quaternary structure nearly as relaxed as oxyhemoglobin does, according to the results mentioned in literature.

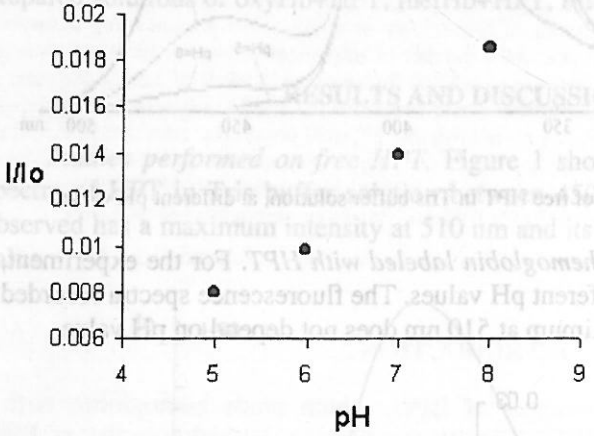

Fig. 4. – The values of I/I_0 ratio as a function of pH buffer solution for cyanHb labeled with HPT.

Studies performed on methemoglobin labeled with HPT. Unlike in the previous cases we noticed a higher increase of fluorescence intensity with the increasing of pH, so a strong Bohr effect is mentioned. We conclude that methemoglobin exists in a more strained state than oxyhemoglobin and cyanhemoglobin.

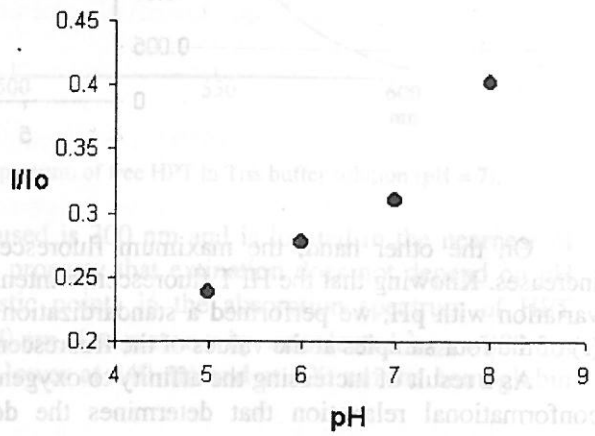

Fig. 5. – The values of I/I_0 ratio as a function of pH buffer solution for metHb labeled with HPT.

Studies performed on fluorhemoglobin labeled with HPT. The experimental results presented in Figure 6 allow us to conclude that fluorhemoglobin has the most strained T state.

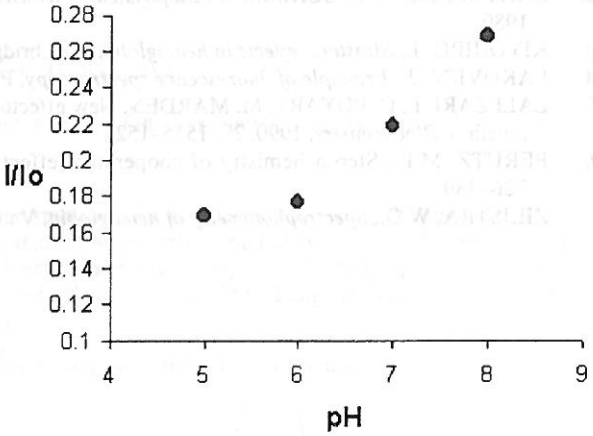

Fig. 6. – The values of I/I_0 ratio as a function of pH buffer solution for fluorHb labeled with HPT.

CONCLUSIONS

This paper surveys a method of indirect fluorescence labeling of hemoglobin, referring to the fact that the heme is a strong quencher of any fluorophor used to label this molecule directly. Use was made of both the property of allosteric effector of HPT and the modulator property of the hemoglobin quaternary structure induced by pH. The fraction of HPT molecules not bound to hemoglobin was measured, as the bound ones have their fluorescence quenched by the heme. We have always used the difference between total HPT concentration and the concentration of bound HPT.

As regards the oxyhemoglobin labeled with HPT, we state that the alkaline Bohr effect is observed within the range of chosen pH values. It can be seen that the values of I/I_0 ratio for cyanhemoglobin are very close to the values obtained for oxyhemoglobin. This indicates that cyanhemoglobin quaternary structure is nearly as relaxed as that of oxyhemoglobin. For methemoglobin: unlike in the previous cases, a higher increase of the fluorescence intensity was noticed with the pH value, increasing that signifies a stronger Bohr effect. We can conclude that methemoglobin exists in a higher strained state than oxyhemoglobin and cyanhemoglobin. The data analyzed until now lead us to conclude that fluorhemoglobin presents the most strained T state among all derivatives of hemoglobin presented in this paper.

It is consequential to identify and localize the binding site of HPT because it could provide the possibility of obtaining information as regards the surfaces of the molecular region located near this site.

REFERENCES

1. BETTATI, S., A. MOZZARELLI, T-State hemoglobin binds oxygen non-cooperatively with Bohr effect, *J. Biol. Chem.*, 1998, **272**, 32050–55.
2. CANTOR, C.R., P.R. SCHIMMEL, *Biophysical Chemistry*, Freeman W.H. and Co., New York, 1980.
3. KIYOHIRO, I., *Allosteric effects in hemoglobin*, Cambridge University Press, 1982.
4. LAKOVICZ, J., *Principle of fluorescence spectroscopy*, Plenum Publ. Co., 1983.
5. LALEZARI, I., C. POYART, M. MARDEN, New effectors of human hemoglobin: structure and function, *Biochemistry*, 1990, **29**, 1515–1523.
6. PERUTZ, M.F., Stereochemistry of cooperative effects in hemoglobin, *Nature*, 1970, **228**, 726–739.
7. ZILJSTRA, W.G., *Spectrophotometry of hemoglobin*, Van Gercum, Amsterdam, 1980.