RESEARCH ARTICLE

Interaction of glycated myoglobin with trifluoperazine: A biophysical elucidation of the consequences

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Abstract

Affinity of a protein towards a drug depends on its structural configuration. High concentration of glucose as occurred in diabetes mellitus interacts with proteins and other molecules. Glycation of protein interferes the structure, resulting modification in its interaction profile with drugs. Trifluoperazine (TFZ), a phenothiazine group of drugs is used widely for the treatment of psychopathy. It is reported that interaction of TFZ with hemoglobin is largely affected by the glycation of the later. Myoglobin (Mb), another heme protein is identical to hemoglobin in many respects and comes out of the muscle to the blood stream during vigorous exercise. As a result, myoglobin also gets glycated in diabetic patients. However, the interaction profile of TFZ-Mb upon glycation has not been established yet. With this view, here a comparative study on the interaction of TFZ with Mb and glycated myoglobin (GMb) is reported. The study is purely based on spectrofluorimetric analysis. The binding parameters like, binding affinity constant and binding sites as well as the nature of the interactions are studied. It is concluded that the affinity of myoglobin is significantly increased upon glycation, however, the number of binding sites and the nature of interaction remain unaffected.

Keyword: Trifluoperazine, TFZ, myoglobin, glycation, drug-protein interaction, fluorometry.

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Introduction

Drug-protein interaction plays a crucial role in the transport and bioavailability of different molecules in the physiological system. Many researchers have motivated interdisciplinary research, preferably to ascertain the biological function of both synthetic and analytical molecules upon interaction with different biomolecules especially protein. In this context, blood proteins endorse the most active and assorted roles in cells by delivering diverse functions including the control of biochemical reactions, providing intracellular and extracellular scaffolding, transport of different biomolecules, controlling enzymatic action, and building the immune system (Hegyi & Gerstein, 1999). Since proteins are the preferable targets for drugs, they represent the best model for in vitro characterization of pharmacological action of therapeutic drugs (Chatterjee & Suresh Kumar, 2016). Prominent among such are globular proteins like albumin, hemoglobin and myoglobin.

Trifluoperazine (TFZ) is a well-known and widely used potent phenothiazine drug. The compound possesses an aromatic side chain, commonly termed piperazine moiety (Figure 1). Phenothiazines with aromatic side chains are quite potent antipsychotic agents. At higher doses it prevents psychiatric illness, however, at lower doses, it can be used for management of vomiting and nausea (Tardy et al., 2014). TFZ, after its development in 1950s, was introduced in the year of 1959 (Howland, 2016). It is an approved drug for the treatment of schizophrenia as well as other non-psychotic anxiety (Howland, 2016). It has high affinity to directly bind with calmodulin and shows inhibitory role on the later (Vandonselaer et al., 1994). TFZ is also used to prevent some forms of lung cancer (Abdulrezzak, 2016) and suppress tumor growth (Kang et al., 2017). However, we have observed that TFZ interacts with globin part of hemoglobin, and the interaction is positively cooperative, electrostatic and exothermic in nature, whereas the drug interacts with myoglobin (Mb) in non-cooperative, hydrophobic and endothermic fashion (Bhattacharya et al., 1994, 1996). The difference in the binding behavior can be attributed to the subunit interaction property present and absent in tetrameric hemoglobin and monomeric Mb, respectively. As a result of interaction with phenothiazines, more oxygen is released from both oxyhemoglobin and oxyMb. Our report suggests that NaCl-induced alteration in the quarternary structural organization of hemoglobin influences its binding mobility with phenothiazines (Bhattacharya et al., 1998). We have also shown that glycated hemoglobin (HbA_{1c}), as found in high quantity in diabetic patients, upon interaction with TFZ has evidenced decreased oxygen release, lower binding affinity and cooperativity, and increased heme loss in comparison to non-glycated hemoglobin (HbA_0) (Kar et al., 2006). Furthermore, we have shown that glycation of myoglobin induces structural and functional modifications of the protein

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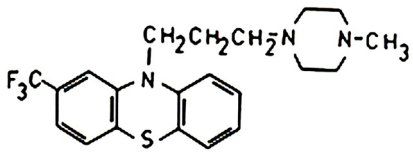

Figure 1: 2-trifluoromethyl-10-[3'-(1- methyl-4-piperazinyl) propyl] phenothiazine or TFZ.

(Roy et al., 2004; 2010). Thus, like hemoglobin, glycation is also expected to influence the interaction of myoglobin with TFZ. However, no such report is available so far.

Myoglobin (Mb) is a heme-containing protein involved in the storage and transfer of oxygen within muscle cells (Stryer, 1995). It is an extremely compact protein with eight alpha-helices and five non-helical segments between helical regions. Since muscle cells possess insulin-dependent glucose transport processes, glucose concentrations within the cell is low in diabetic condition due to deficiencies or inactive insulin. However, vigorous repeated exercise produces muscle damage, releasing myoglobin in circulation (Halliwell and Gutteridge, 2000; Cooper *et al.,* 2002). The content of myoglobin increases from 1.2 ± 0.1 mg/kg up to 2.6 ± 0.1 mg/kg after single intensive physical exercise in rat blood (Chaikovskii *et al.,* 1987); thereby enhances the risk of myoglobin glycation in the circulation. Thus, with this view, it is wiser to study the interaction dynamics of glycated myoglobin with TFZ and its possible consequences using different biophysical and biochemical tools.

Materials

Horse heart myoglobin (Mb), trifluoperazine (TFZ), sephadex G25, were purchased from Sigma Chemical Co, USA. Bio-Rex70 resin (200 – 400 mesh) was purchased from BioRad, India. Other chemicals were of analytical grade and purchased locally.

Methods

Preparation of TFZ solution

Aqueous solution of TFZ was made fresh before each set of experiment. For this purpose, a certain amount of the drug was weighed and dissolved in distilled water. The absorption spectrum (220 – 400 nm) of the drug was checked and found similar with that of Bhattacharya et al. (1994, 1996). The concentration of the drug solution was determined using its molar extinction coefficient at 305 nm, i.e., $\varepsilon_{305\ nm} = 3162\ M^{-1}cm^{-1}$ (Budavari *et al.*, 1989).

Glycation of Myoglobin (Mb)

Glycation of Mb was performed *in vitro* following the method of hemoglobin glycation as described by Cohen and Wu (1994). Mb solution was prepared by dissolving the protein in 50 mM potassium phosphate buffer (PB), pH 6.6 for 24 hr at 4 °C. The Mb solution of 5 mg/ml concentration was incubated in stoppered glass vials with different glucose concentrations (65, 125, 166, 248 and 330 mg/dl) along with control (without glucose) for 6 days at 25 °C. Mb and glucose solutions were sterilized separately by filtration before addition. The additions were made under aseptic condition (Roy et al., 2004). The amount of glucose consumed after incubation was determined from the estimation of free glucose left in the samples following the glucose oxidase method (Trinder, 1969).

Separation of glycated and non-glycated fractions

After in vitro glycation, glycated myoglobin (GMb) and unchanged non-glycated myoglobin (Mb) present in the reaction mixture were separated by ion exchange chromatography using Biorex-70 resin column (7 cm × 1.0 cm), pre-equilibrated with 50 mM PB, pH 6.0. Two protein fractions, fraction-I and fraction-II were eluted with elution buffers, 50 mM PB, pH-6.6 and 7.0 respectively. After separation, fraction-II was applied to Sephadex G25 column (7 cm × 1.0 cm), pre-equilibrated with 50 mM PB, pH 6.6, so that both fractions were collected in the same buffer (Roy et al., 2004). The concentrations of the fractions were determined using extinction coefficient $\varepsilon_{408\ nm} = 116\ mM^{-1}\ cm^{-1}$ (Wittenberg and Wittenberg, 1981).

Confirmation of glycation in separated fractions

Glycation in fractions were tested by TBA reaction according to the method developed by Fluckiger and Winterhalter (1976) for testing glycation in hemoglobin. One milliliter each of 10 µM separated fractions was mixed with 0.5 ml 0.3 N oxalic acid and heated for 1 hr in a boiling water bath. After cooling to room temperature, 0.5 ml of 40 % TCA was added and the resulting precipitate was removed by filtration. Half a milliliter of 0.05 % TBA was added to the supernatant and the mixture was incubated at 40 °C for 30 min. The coloured chromophore gave a characteristic absorption maximum around 443 nm, indicating glycation reaction. The isolated protein fractions thus detected as glycated (GMb) and non-glycated (Mb) species of myoglobin were subjected to characterization (Roy et al., 2004).

Interaction of Mb and GMb with TFZ

All fluorescence measurements have been done in Hitachi F3010 spectrofluorimeter using 1 cm pathlength quartz cuvette. Excitation band width was kept at 5 nm, whereas emission band width was kept at 10 nm. Drug (up to 40 µM) from a concentrated stock solution was added to 3 ml Mb or GMb solution by micropipette, so that the volume increment in the cuvette was negligible. When excited at 285 nm, the emission maxima of Mb and GMb appeared at 334.2 nm and 338 nm, respectively. Quenching of the tryptophan fluorescence intensities of Mb and GMb in the presence of the added drug was measured from the change of the respective emission intensity at the emission maxima. Since TFZ has very low absorbance (an absorption minimum) at 285 nm, it does not contribute significantly to the fluorescence emission, when excited at 285 nm. The binding affinity constant (K) was calculated from the linear plot of 1/ΔF vs. 1/Lt, following

the equation of Kapp *et al.* (1990).

$$\frac{1}{\Delta F} = \frac{1}{\Delta F_{max}} + \frac{1}{K.L_t} \times \frac{1}{\Delta F_{max}}$$

Where $\Delta F = F_0 - F$; F_0 and F represent the fluorescence intensities of Mb and GMb at 332 nm in the absence and presence of the total drug concentration (L_t), respectively. ΔF_{max} is the maximum change in the fluorescence intensities. The intercept of the plot on $1/\Delta F$ axis extrapolated to $1/L_t = 0$ measures $1/\Delta F_{max}$ and the slope gives the estimate for the binding affinity constant, K.

The number of binding sites (p) was estimated from the linear plot of $1/(1-\theta)$ vs L_t/θ following the equation of Kapp *et al.*, 1990.

$$\frac{1}{1-\theta} = \frac{K.L_t}{\theta} - K.p.A_t$$

Where, A_t is the fixed concentration of Mb or GMb, and $\theta = \Delta F/\Delta F_{max}$ measures the extent of binding, and K is the binding affinity constant.

Nature of interaction of TFZ with Mb and GMb

Fluorescence quenching of Mb or GMb was measured by gradual addition of TFZ in presence of different NaCl concentrations (0.02 – 0.3 M). Binding affinity constant, K was estimated from the extents of quenched fluorescence of the proteins to find out the nature of interaction.

Result and Discussion

Interaction of TFZ with Mb and GMb was studied by using spectrofluorimetric technique. When excited at 285 nm, the emission maximum for GMb appeared to be 333.6 nm and that of Mb was 331.6 nm. This difference in emission maximum may be due to glycation-induced structural modification of the protein. With gradual addition of TFZ to the proteins (Mb or GMb), the fluorescence intensity of Mb or GMb decreased and emission maxima shifted slightly towards longer wavelength, suggesting the drug-protein interactions. For Mb-TFZ interaction (Figure 2a), the wavelength shift was from 331.6 nm to 335 nm and for GMb-TFZ interaction (Figure 2b) the wave length shift was from 333.6 nm to 338.6 nm for drug concentrations from 0 to 40 µM. The binding

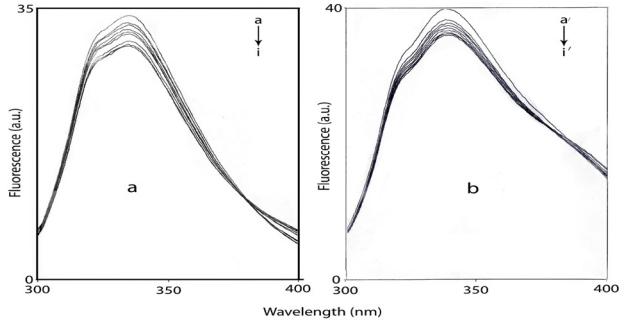

Figure 2: Representative fluorescence emission spectra of 8 µM Mb (a) and GMb (b) in the absence and presence of increasing concentrations of added TFZ. Total concentrations of TFZ used were 0, 5, 10, 15, 20, 25, 30, 35 and 40 µM and respective spectra were presented for Mb -TFZ interaction (a to i) and GMb-TFZ interaction (a/ to i/) (a.u. is arbitrary unit).

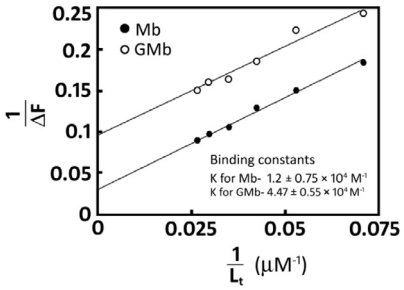

Figure 3: Representative plots for $1/\Delta F$ versus $1/L_t$ for the determination of binding affinity constants (K) of Mb-TFZ and GMb-TFZ inter- actions. $\Delta F = F_0 - F$, where F_0 and F are fluorescence intensities of 8 µM Mb or GMb at 332 nm in the absence and presence of TFZ concentrations (L_t), respectively.

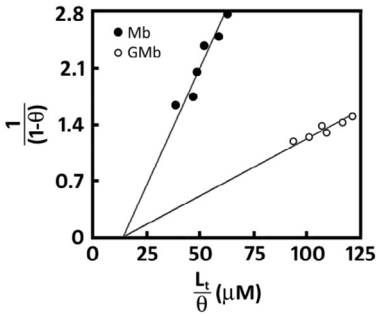

Figure 4: Representative plots for $1/(1-\theta)$ versus Lt/θ for the determination of binding sites of Mb- TFZ and GMb-TFZ interactions. $\theta = \Delta F/\Delta Fmax$ where, $\Delta Fmax$ is the maximum change in fluorescence intensities in the presence of total drug (Lt).

constant (K) was calculated from the linear plots of $1/\Delta F$ vs $1/L_t$ (Figure 3) following the equation of Kapp et al. (1990), as described in method section. The binding affinity constants for TFZ interacting to Mb and GMb in 50 mM PB buffer, pH 6.6 appeared to be $1.2 \pm 0.75 \times 104$ M^{-1} and $4.47 \pm 0.55 \times 104$ M^{-1}, respectively. Glycation is thus responsible for increased binding affinity constant for interaction with TFZ. In a previous study, Bhattacharyya et al. (1998) have reported that the possible site for interaction of CPZ, another phenothiazine drug is around tryptophan residues of hemoglobin. Glycation induces exposure of tryptophan residues in Mb, as shown by treatment with quencher like acrylamide and KI (Roy et al, 2010). Increased binding affinity constant of TFZ-GMb interaction in comparison with that of TFZ-Mb interaction might therefore be correlated with more surface accessible tryptophan residues in the glycated protein. Number of binding sites involved in the interaction of TFZ with Mb or GMb was estimated from the spectrofluorometric emission spectra using the linear plot of $1/(1-\theta)$ vs L_t/θ **(**Figure 4) following the equation of Kapp et al., 1990. The number of binding sites (p) for both Mb and GMb appeared to be 2 ± 0.3. Mb has two tryptophan residues and glycation may alter the structure of Mb by exposing buried tryptophan residues (Roy et al., 2010). Although, the number of binding sites did not change in the glycated protein, the modification in glycated one might have led to the increased binding affinity constant. Binding of Mb and GMb with TFZ was studied at varying concentrations of NaCl ranging from 0.02 to 0.3 M. It

Table 1: Binding affinity constants of Mb and GMb to TFZ at different NaCl concentrations

[NaCl], (M)	*Binding affinity constants*	
	Mb (M^{-1})	*GMb (M^{-1})*
0.02	$12.5 \pm 1.2 \times 10^3$	$5.6 \pm 0.9 \times 10^3$
0.075	$13.7 \pm 1.5 \times 10^3$	$4.0 \pm 0.85 \times 10^3$
0.15	$13.5 \pm 1.2 \times 10^3$	$5.0 \pm 0.9 \times 10^3$
0.3	$13.7 \pm 1.8 \times 10^3$	$3.7 \pm 1.2 \times 10^3$

was found that the binding constants of both Mb and GMb did not vary significantly with increasing NaCl concentration (Table 1), indicating hydrophobic nature of the interactions for both the proteins.

Conclusion

Both Mb and GMb interact with phenothiazine drug, like TFZ. Binding affinity for TFZ is higher in glycated myoglobin than non-glycated one. However, the number of binding sites appears to be same in both proteins. Glycation may cause structural modification of myoglobin, which, in turn, alters its interaction with drug like TFZ.

Conflict of Interest

Author has no conflict of interest to disclose.

References

Abdülrezzak Ü, Erdoğan Z, Silov G, Özdal A, and Turhal Ö (2016): Effect of trifluoperazine on Tc-99m sestamibi uptake in patients with advanced nonsmall cell lung cancer, *Indian J Nucl Med*, **31,** 103–107. doi: 10.4103/0972-3919.178256

Bhattacharya J, Bhattacharya M, Chakraborti AS, Choudhury U and Podder RK (1994): Interaction of chlorpromazine with myoglobin and hemoglobin: A comparative study, *Biochem Pharmacol,* **47,** 2049-2053.

Bhattacharya J, Bhattacharya M, Chakraborti AS, Choudhury U and Podder RK (1998): Structural organization of hemoglobin and myoglobin influence their binding with phenothiazines, *Int J Biol Macromol,* **23,** 11-18.

Bhattacharyya J, Bhattacharyya M, Chakraborti AS, Choudhury U and Podder RK (1996): Trifluoperazine is more effective than chlorpromazine in releasing oxygen from haemoglobin and myoglobin, *J Pharm Pharmacol,* **48,** 965-967.

Budavori S, Ó Neil MJ, Smith A and Heckelman P (1989): In: *Merk Index*, 11th edn, Merk and Co Inc, USA, p. 9599.

Chaikovskii VS, Basharina OB, Shaliapina IV and Rogozkin VA (1987): Effect of physical exercise on myoglobin and tropomyosin levels in skeletal muscles and myoglobin level in rat blood, *Vopr Med Khim,* **33,** 79-83.

Chatterjee S and Suresh Kumar G (2016): Binding of fluorescent acridine dyes acridine orange and 9-aminoacridine to hemoglobin: Elucidation of their molecular recognition by spectroscopy, calorimetry and molecular modeling techniques, *Journal of Photochemistry and Photobiology B: Biology,* **159,** 169-178. https://doi.org/10.1016/j.jphotobiol.2016.03.045.

Cohen MP and Wu V (1994): Purification of glycated hemoglobin, *Methods Enzymol,* **231,** 65-75.

Cooper CE, Vollard NB, Choueiri T and Wilson MT (2002): Exercise, free radicals and oxidative stress, *Biochem Soc Trans,* **30,** 280-285.

Fluckiger R and Winterhalter KH (1976): In vitro synthesis of hemoglobin A_{1c}, *FEBS Lett,* **71,** 356-360.

Halliwell B and Gutteridge JMC (2000): In: *Free Radicals in Biology and Medicine,* Oxford University Press, New York, pp. 105-245.

Hegyi H and Gerstein M (1999): The relationship between protein structure and function: a comprehensive survey with application to the yeast genome, *Journal of molecular biology,* **288**(1), 147-164.

Howland RH (2016): Trifluoperazine: A Sprightly Old Drug, *Journal of Psychosocial Nursing and Mental Health Services*, **54**, 20-22. DOI: 10.3928/02793695-20151223-01.

Kang S, Hong J, Lee JM, Moon HE, Jeon B, Choi J, Yoon NA, Paek SH, Roh EJ, Lee CJ and Kang SS (2017): Trifluoperazine, a Well-Known Antipsychotic, Inhibits Glioblastoma Invasion by Binding to Calmodulin and Disinhibiting Calcium Release Channel IP3R, *Cancer Biology and Signal Transduction*, DOI: 10.1158/1535-7163.MCT-16-0169-T.

Kapp EA, Daya S, Whiteley CG (1990): Protein-ligand interactions: interaction of nitrosamines with nicotinic acetylcholine receptor, *Biochem Biophys Res Commun*, **167**(3), 1383-92. doi: 10.1016/0006-291x(90)90676-e.

Kar M, Roy A, Bose T and Chakraborti AS (2006): Effect of glycation of hemoglobin on its interaction with trifluoparazine, *Protein J,* **25,** 202-211.

Roy A, Sen S and Chakraborti AS (2004): *In Vitro* Nonenzymatic Glycation Enhances the Role of Myoglobin as a Source of Oxidative Stress, *Free Radical Research,* **38,** 139-146.

Roy A, Sil R and Chakraborti AS (2010): Non-enzymatic glycation induces structural modifications of myoglobin, *Mol Cell Biochem,* **338**, 105–114. DOI 10.1007/s11010-009-0343-7

Stryer L (1995): In: *Biochemistry,* 4th edn. Eds. WH Freeman, New York, pp. 147-178.

Tardy M, Dold M, Engel RR, Leucht S (2014): Trifluoperazine versus low-potency first-generation antipsychotic drugs for schizophrenia, *Cochrane Database Syst Rev,* doi: 10.1002/14651858.CD009396.pub2.

Trinder P (1969): Determination of blood glucose using an oxidase-peroxidase system with a noncarcinogenic chromogen, *J Clin Pathol,* **22,** 158-161.

Vandonselaar M, Hickie R, Quail W and Delbaere LTJ (1994): Trifluoperazine-induced conformational change in Ca2+-calmodulin. *Nat Struct Mol Biol,* **1**, 795–801. https://doi.org/10.1038/nsb1194-795

Wittenberg JB and Wittenberg BA (1981): Preparation of myoglobin, *Methods Enzymol,* **76,** 29-4.