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Research Article
Reactive Sulphydryl Groups in Horse Carbonmonoxyhaemoglobin

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Abstract

Background and Objective: Reactive sulphydryl groups in haemoglobin has been related with oxygen binding. In this study, the researchers analyzed the reactive sulphydryl groups in horse (*Equus ferus caballus*) carbonmonoxyhaemoglobin. **Materials and Methods:** Hemolysate gotten from fresh horse blood was converted to yield the carbonmonoxy derivative. It was then separated via carboxymethylcellulose into major and minor fractions of haemoglobin. These fractions were titrated with Ellman's reagent (DTNB) and p-hydroxymercuri(II)benzoate (p-MB) stock solutions in increasing volumes. **Results:** Results showed that two sulphydryl groups reacted with DTNB and p-MB in both major and minor haemoglobin fractions. p-MB is known to be more reactive with thiols than DTNB, on the other hand the reactivity is the same with horse carbonmonoxyhaemoglobin. **Conclusion:** This study will enable a better understanding as regards the kinetics and equilibrium behind this reaction.

Key words: *Equus ferus caballus*, sulphydryl groups, carbonmonoxyhaemoglobin, DTNB, p-MB, hemolysate

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Haemoglobin is a metalloprotein which is found in vertebrates with a primary function of oxygen transport[1]. It confers high capacity protection in erythrocytes against reactive oxygen and nitrogen species (ROS and RNS) as a result of the sulphydryls present, which act as antioxidants[2,3]. Apart from sulphydryls located in the interior of haemoglobin, they are classified into two categories. Firstly, those with high ease of access to solvent and whose conformational alteration in the haemoglobin tetramer does not affect its microenvironment[4]. Secondly, those which display oxygen linked reactivity[5]. The number of reactive sulphydryl groups in haemoglobin with sulphydryl reagents is generally less than the total number of cysteines in the molecule. This phenomenon occurs as a result of masked thiols and sulphdryl reagent used[6,7]. Oxygen affinity and reactivity of sulphydryl group are linked[8], as the reactivity of thiols found in haemoglobin are inversely correlated with their oxygen affinity[9]. Sequenced horse genome shows 53% synteny with humans[10]. Giardina *et al.*[11] reported on organic phosphates (2,3-BPG) and chloride effects on the properties of horse haemoglobin. There are a few reports on the properties of horse blood[11] and no comprehensive study has been performed on the reactivity of horse haemoglobin towards organic and inorganic ions. This prompted us to study the number of reactive sulphydryl groups in horse (*Equus ferus caballus*) carbonmonoxyhaemoglobin. The total number of reactive sulphydryl groups was found at the end of the study.

MATERIALS AND METHODS

Preparation of haemoglobin: Horse blood was obtained by drawing blood from the jugular vein by a certified veterinary doctor. This was collected into heparin bottles to avoid coagulation. Haemoglobin was prepared as previously described by Okonjo *et al.*[8].

Preparation of carbonmonoxyhaemoglobin: The oxyhaemoglobin prepared was converted to the carbonmonoxy derivative by bubbling carbon monoxide through it for about 20 min according to the method of Antonini and Brunori[12].

Separation of horse haemoglobins: Hemolysate was separated into major and minor haemoglobin as previously described by Okonjo *et al.*[8]. Haemoglobin fractions were then converted to the carbonmonoxy derivative by passing carbon monoxide into each of the fractions for about 20 min. The dialysis of the haemoglobin solutions to remove phosphate ions were repeated twice in 5 dm^3 (pH = 7.0) dialysis solutions. The haemoglobin fractions were stored in a freezer as carbonmonoxy derivatives and were divided into small portions. A portion was thawed at a time for use. This was to ensure that fresh haemoglobin was used throughout the experiments. The organic phosphate 2,3-biphosphoglycerate (2,3-BPG) exists with haemoglobin in the red blood cell. Since 2,3-BPG is not easily removed from haemoglobin by dialysis, each haemoglobin solution was passed through a mixed bed ion exchange column as described by Dintzis[13] to remove endogenous 2,3-BPG and excess ions.

Sulphydryl titration with Ellman's reagent (DTNB): The sulphydryl titration of horse carbonmonoxyhaemoglobin was done according to Boyer's method[14], as previously reported for dog haemoglobin[6,14]. About 10 cm^3 of 10 μmol dm^{-3} (haem) of horse carbonmonoxyhaemoglobin solution in phosphate buffer pH 7.6 was accurately measured into several clean, dry test tubes. Increasing volumes of stock DTNB was added to the different test tubes. These mixtures were stirred and left to equilibrate for 3 h. The absorbance of each solution was read at 412 nm. The absorbance (A_{412}) was corrected for dilution and for the absorbance of DTNB to obtain ΔA_{412}. The concentration of 5-thio-2-nitrobenzoate (TNB) produced as calculated from the change in absorbance, assuming a molar absorption coefficient of 14,000 mol^{-1} dm^3 cm^{-1} for TNB. The ratios of TNB concentration to the concentration of haemoglobin tetramer were plotted against the volume of DTNB. This experiment was repeated in triplicate and the mean number of titratable sulphydryl groups was calculated.

Sulphydryl titration with p-hydroxymercuri(II)benzoate (p-MB): The sulphydryl titration of horse carbonmonoxyhaemoglobin was done according to Boyer's method[14], as previously reported for dog haemoglobin[6,15]. About 3 cm^3 of 10 μmol dm^{-3} (haem) of horse carbonmonoxyhaemoglobin solution in phosphate buffer pH 7.8 was accurately measured into several clean, dry test tubes. Increasing volumes of stock p-MB was added to the different test tubes. These mixtures were stirred and left to equilibrate for 3 h. The absorbance of each solution was read at 250 nm. The absorbance (A_{250}) was corrected for dilution and for the absorbance of p-MB to obtain ΔA_{250}. The concentration of parahaemoglobinmercuri(II)benzoate (p-HbSMB) produced as calculated from the change in absorbance, assuming a molar absorption coefficient of 7,600 mol^{-1} dm^3 cm^{-1} for p-HbSMB. The ratios of p-HbSMB concentration to the concentration of haemoglobin tetramer were plotted against the volume of p-MB. This experiment was repeated in triplicate and the mean number of titratable sulphydryl groups was calculated.

RESULTS

Figure 1 and 2 show results of the titration of the major and minor horse carbonmonoxyhaemoglobin with Ellmans reagent (DTNB). The plot of [TNB]/[Hb_4], moles of TNB complex formed per mole of haemoglobin tetramer, against the volume of DTNB gave an average of 1.82±0.03 and 2.19±0.03 reactive sulphydryl groups per haemoglobin tetramer,

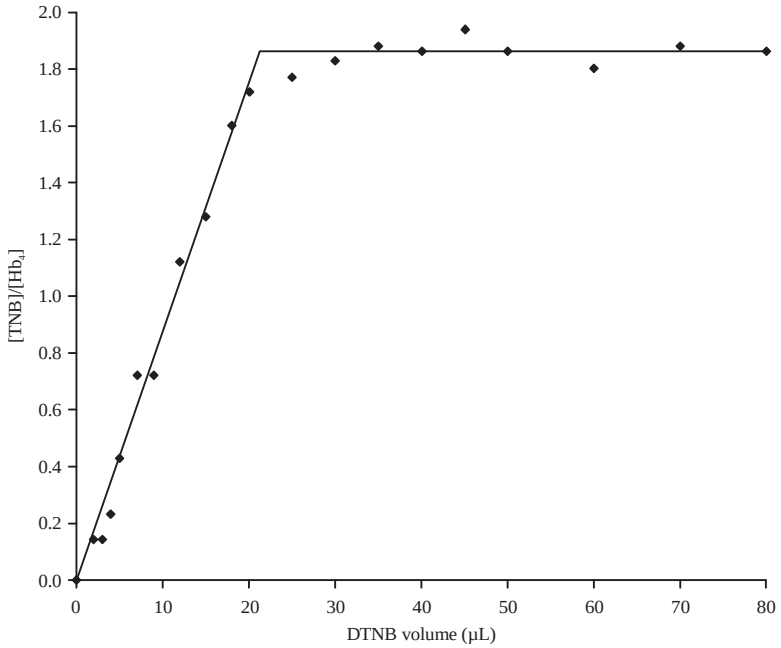

Fig. 1: Graph of titration of the major horse carbonmonoxyhaemoglobin with DTNB as a result of ratio of the concentration of complex produced per haemoglobin tetramer (Hb_4) as a function of the volume of the DTNB mixed with 10 cm^3 of haemoglobin

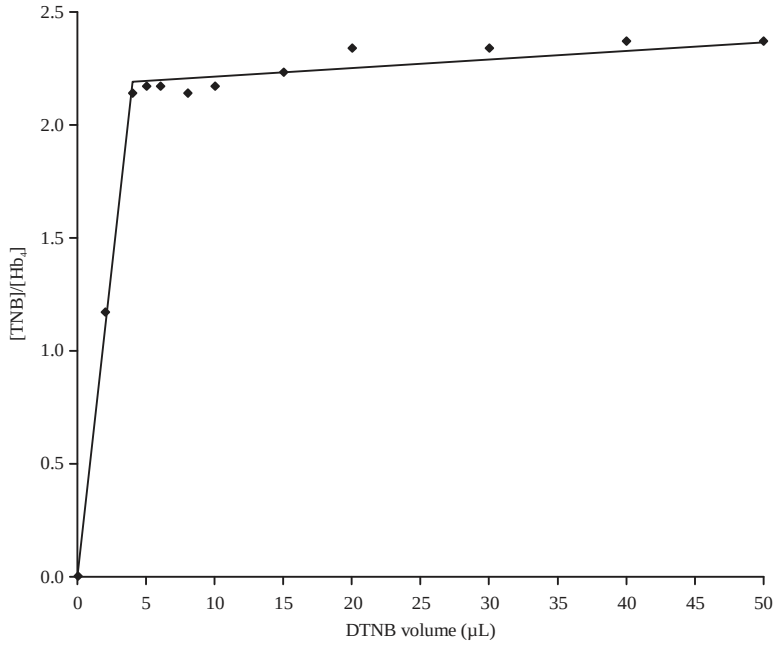

Fig. 2: Graph of titration of the minor horse carbonmonoxyhaemoglobin with DTNB as a result of ratio of the concentration of complex produced per haemoglobin tetramer (Hb_4) as a function of the volume of the DTNB mixed with 3 cm^3 of haemoglobin

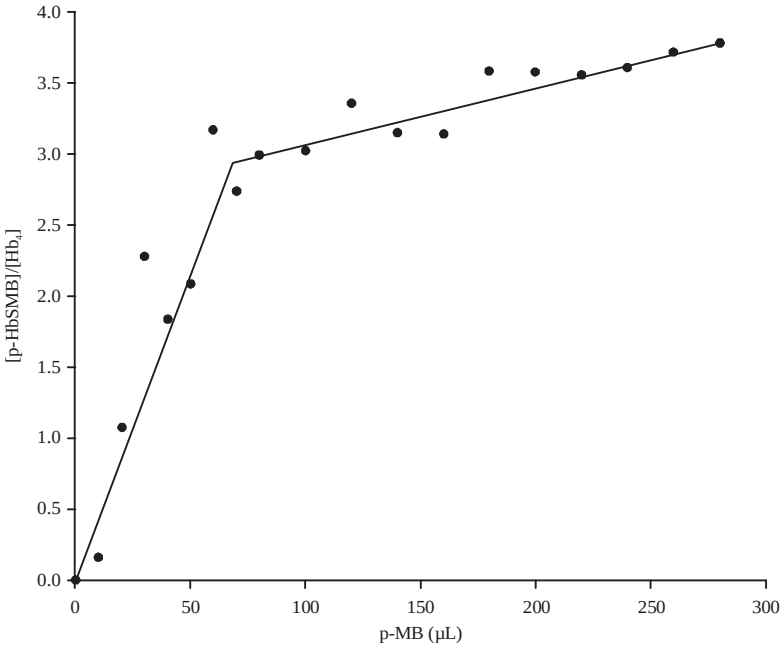

Fig. 3: Graph of titration of the major horse carbonmonoxyhaemoglobin with p-MB as a result of ratio of the concentration of complex produced per haemoglobin tetramer (Hb_4) as a function of the volume of the p-MB mixed with 3 cm^3 of haemoglobin

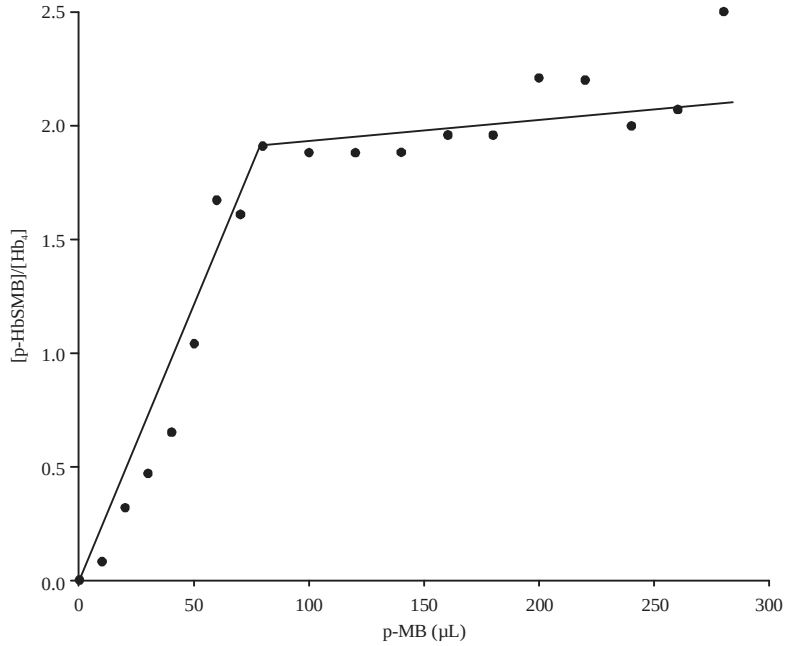

Fig. 4: Graph of titration of the minor horse carbonmonoxyhaemoglobin with p-MB as a result of ratio of the concentration of complex produced per haemoglobin tetramer (Hb_4) as a function of the volume of the p-MB mixed with 10 cm^3 of haemoglobin

respectively. This indicates that two out of the four sulphydryl groups per molecule in the major and minor horse haemoglobin are accessible to DTNB.

Figure 3 and 4 show results of the titration of the major and minor horse carbonmonoxyhaemoglobin with p-hydroxymercuri(II)benzoate (p-MB). The plot of

[p-HbSMB]/[Hb_4], moles of parahaemoglobinmercuri(II) benzoate complex formed per mole of haemoglobin tetramer, against the volume of p-MB gave an average of 2.61±0.07 and 1.87±0.03 reactive sulphydryl groups per haemoglobin tetramer respectively. This indicates that two out of the four sulphydryl groups per molecule in the major and minor horse haemoglobin are accessible to p-MB.

DISCUSSION

Kleinschmidt and Sgouros[7] in their review reported four sulphydryl groups per tetramer in both major and minor horse haemoglobins amino acid sequences. These groups are located at position CysF9[93] of each of the two β-chains and at position CysG11[104] of each of the two α-chains[16]. In each of the two haemoglobin types, only two sulphydryl groups were detectable by titration with DTNB and p-MB as reported by Iheagwam *et al.*[17], Okonjo and Adejoro[15], Okonjo *et al.*[6] and Adeogun[18] using horse, dog and sheep haemoglobin respectively. Sulphydryl groups present in protein react with mercurials, except in cases where they are masked. Masked sulphydryls are known to be unreactive towards any non mercurial reagent as reported by studies[19,20]. Okonjo *et al.*[6] and Kleinschmidt and Sgouros[7] reported that CysG11[104]α located at the $a_1\beta_1$ subunit interface of the α-chain of haemoglobin is masked. It is situated in a negatively charged microenvironment making the dielectric constant less than water. This will cause a significant raise in pKa of the thiol making a nucleophilic attack on the thiol anion of the CysG11[104]α sulphydryl group by the mercurial unable to take place and therefore shows no reaction[6]. It can be deduced that the sulphydryl groups reacting with DTNB and p-MB may be those at position F9[93] on each of the two β-chains as a result of them being free.

CONCLUSION

From this study, two sulphydryl groups reacts with DTNB and p-MB in both major and minor haemoglobin. Kinetic studies to further understand the complete reactivity and reversibility of DTNB's reactivity with haemoglobin sulphydryl group and its equilibrium is being studied in our lab. Tertiary conformational transition reaction should be studied if an allosteric transition occurs on reaction with DTNB and p-MB binding. In addition, oxygen binding should be carried out on these horse haemoglobins to ascertain the link between reactive sulphydryls and oxygen affinity.

SIGNIFICANT STATEMENT

- This study showed the carbonmonoxy derivative of horse haemoglobin has two reactive thiols when spectrophotometrically titrated with mercurials
- Unlike other animal haemoglobins which have more thiols reacting with p-hydroxymercuri(II)benzoate (p-MB) than Ellman's reagent (DTNB), however, horse haemoglobin has the same number of thiols reacting with both thiol reagents
- The study adds to the body of knowledge and enlightens readers concerning the reactive property of horse haemoglobin towards thiol reagents

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