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ORIGINAL ARTICLE

Kinetics of reaction of 5,5′-dithiobis(2-nitrobenzenzoate) with CysF9[93]β sulphydryl group of sheep haemoglobins at neutral and physiological pH ranges

Abideen Idowu Adeogun *

Department of Chemistry, University of Agriculture, Abeokuta, Nigeria

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Abstract The kinetics of the reaction of 5,5′-dithiobis(2-nitrobenzoate) (DTNB) with CysF9[93]β sulphydryl group of the sheep haemoglobins were studied at neutral and physiological pH ($6.8 \leqslant pH \leqslant 7.6$) ranges under pseudo first order conditions. Each reaction is of first order with respect to the DTNB concentration. The pH dependence of the observed rate constant of the reaction at neutral and physiological ranges gave a complex trend. The observed rate shows that at neutral pH, the presence of inositol hexakisphossphate (IHP) leads to a relative lowering of the pseudo first order rate constant. Overall, the rate varied among the derivatives as aquomet > oxy > carbomonoxy and the major haemoglobin show a higher reactivity than the minor haemoglobin.

1. Introduction

Haemoglobin has been considered as one of the possible sources of reactive oxygen species (ROS) and the haem-bound iron has also been proposed as a pro-oxidant (Puppo and Halliwell, 1988). Haemoglobin provides erythrocytes with a high capacity for protection against ROS and reactive nitrogen species (RNS) through antioxidant thiols which are largely independent of conformational alterations in the haemoglobin tetramer (Dafre and Reischl, 1998, 2006; Miranda, 2000; Di Simplicio et al., 1998). Apart from those that are totally internal, haemoglobin thiols seem to belong to two categories. The first are those with high solvent accessibility (external i.e., on surface crevice) and second are those whose microenvironment does not change with haemoglobin conformation, e.g. CysH3[125]β of rat and guinea pig haemoglobins, and CysA10[13]β in mouse haemoglobin, which are possibly involved in physiologically relevant redox reactions such as detoxifying functions. CysF9[93]β belongs to the second category, it display oxygen linked reactivity (Rossi et al., 1998; Miranda, 2000) and in this case, steric hindrance plays an important role in its thiol reactivity.

The number of reactive sulphydryl groups in haemoglobin has been determined by spectroscopic titration with sulphydryl reagents. This has been found to be generally less than the total number of cysteines in the molecule since some thiols are buried (masked) i.e. they are located in the subunit contact region

* Tel.: +234 8036126987.
E-mail address: abuaisha2k3@yahoo.com
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between the α_1 and β_1 subunits and hence not accessible. The number of titratable thiol groups in haemoglobin depends on the thiol reagent used (Okonjo et al., 1979). Mercurial reagents titrate all the free thiol groups in haemoglobin while non-mercurial reagents titrate only the thiols which can form the thiolate anion. For example, the major chicken haemoglobin has ten sulphydryl groups. Of these, eight are titratable with p-chloromercuri(II)benzoate (pCMB) in the intact haemoglobin while four are titratable with 5, 5′-dithiobis(2-nitrobenzoate), (DTNB), a non-mercurial reagent (Antonini and Brunori, 1971; Okonjo and Nwozo, 1997). Human haemoglobin A has six sulphydryl groups. Of these, only the two at position F9[93]β of each of the two β chains are titratable with either p-CMB or DTNB. The masked sulphydryl groups are at positions G11[104]α and G14[112]β (Okonjo and Okia, 1993).

The high reactivity of the thiolate anion has placed sulphydryl groups among the most important functional groups to be considered when investigating the reactivities, functions, mechanisms and conformations of macromolecules. The reactivity of protein thiols is determined, besides steric factors, by their degree of nucleophilicity, which is maximised by stabilisation of their thiolate form (Kortemme and Creighton, 1995; Miranda, 2000). The CysF9[93]β sulphydryl group has been employed as an indicator for tertiary and quaternary structural changes in haemoglobin (Okonjo et al., 1989; Okonjo et al., 1995; Okonjo et al., 1996 and Okonjo and Nwozo, 1997). An X-ray structure study of oxyhaemoglobin at 2.1 Å resolution, showed that the CysF9[93]β sulphydryl group exists in two conformations (Shaanan, 1983). In sheep haemoglobin there is a deletion of one amino acid at the beginning of the β chain which is a binding site for organic phosphate that regulates the allosteric behaviour of haemoglobin (Okonjo et al., 2009). The interaction of haemoglobin with organic phosphate is linked to the conformation of the CysF9[93]β hence, this study is aimed at investigating the effect of the deletion of the amino group on the extent of exposure of CysF9[93]β in the neutral and physiological pH ranges in sheep haemoglobin.

2. Materials and methods

5,5′-Dithiobis(2-nitrobenzoate), parahydroxymercuri(II)benzoate (p-MB), and inositol hexakisphosphate were purchased from Sigma Aldrich USA. Phosphate buffer solutions were prepared with reagent grade chemicals made by British Drug Houses [BDH], London. The reagents were used without further purification.

2.1. Preparation of haemoglobin

The blood of sheep that had been duly certified by health officials for consumption at the Bodija Slaughter Slab, Ibadan, was collected with freshly prepared acid-citrate-dextrose anticoagulant upon decapitation. Haemoglobin was prepared from the blood sample using standard laboratory procedures as follows:

The blood sample was centrifuged for 20 min at 5 °C at a speed of 12,000 rpm using an MSE High Speed 18 ultracentrifuge, with an $8 \times 50\ cm^3$ capacity rotor. The red blood cells were washed three times with isotonic saline solution (for sheep blood, 9.5 g NaCl dm^{-3}). After each washing the resulting mixture was centrifuged at 9,000 rpm for 15 min. The sediment (erythrocytes) was lysed by shaking it vigorously with an equal volume of ice-cold distilled water to yield a mixture of haemoglobin solution and red cell debris.

The mixture was centrifuged at 12,000 rpm for 20 min to remove all stromal impurities. Haemoglobin was decanted from the cake of the cell debris and filtered through glass wool, after which sodium chloride (5% wt/vol.) was added. This was left for 20 min at 5 °C in a refrigerator. Haemoglobin was further centrifuged at a speed of 15,000 rpm for 20 min. Low molecular weight impurities contained in haemoglobin were then removed by dialysing it for 3 h in a $5\ dm^3$ flask against distilled water (pH ≈ 7.0) at 5 °C using polyvinyl chloride dialysis tubing. This procedure was repeated two more times but in 10 mM phosphate buffer pH 6.5.

2.2. Separation of sheep haemoglobins

Sheep haemolysate contains two haemoglobins, major and minor, separation was carried out in a cold room at 5 °C on carboxymethyl cellulose by modifying the method described by Taketa et al. (1971). A 3 cm (diameter) by 30 cm column of Whatman CMC-52 carboxymethylcellulose, a microgranular, preswollen cation exchanger, was used. The resin was pre-equilibrated with 10 mmol dm^{-3} phosphate buffer, pH 6.5. The minor haemoglobin was completely eluted with pH 6.5 buffer, whereas the major component remained bound to the resin. The major haemoglobin was eluted at room temperature with 140 mmol dm^{-3} phosphate buffer, pH 7.0. The haemoglobins were stored in the freezer and thawed when required. Prior to use in experiments, each haemoglobin sample was passed through a Dintzis ion exchange column (Dintzis, 1952) to remove endogenous organic phosphates and undesired ions.

3. Experimental

3.1. Determination of available and reactive sulphydryl group

The total number of reactive thiol groups was determined by Boyer titration (Dintzis, 1952; Boyer, 1954) of the carbonmonoxy derivative in phosphate buffer, pH 7.6 (ionic strength 50 mmol dm^{-3}), using p-hydroxymercuri(II)benzoate. The number of sulfhydryl groups reacting with DTNB was determined as previously described (Okonjo and Okia, 1993).

3.2. Kinetics of the reactions of DTNB with CysF9[93]β sulphydryl groups of sheep haemoglobin

The reaction of DTNB with CysF9[93]β sulphydryl groups of various derivatives of sheep haemoglobin was monitored at 412 nm on a Cecil BioQuest UV–Visible spectrophotometer coupled with the online data acquisition system. The reaction was carried out under pseudo-first order conditions, with DTNB in at least 30-fold excess over the concentration of the reactive sulfhydryl groups. Each run was repeated at least three times under the same experimental conditions and was allowed to proceed to near completion. The data were analysed with a 1990 update of DISCRETE, a computer programme for the analysis of multiple exponential signals (Provencher, 1976a; Provencher, 1976b). A slight modification of DISCRETE