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Research paper

Fetal hemoglobin is much less prone to DNA cleavage compared to the adult protein

Sandeep Chakane, Tiago Matos, Karin Kettisen, Leif Bulow*

Pure and Applied Biochemistry, Department of Chemistry, Lund University, Lund 22362, Sweden

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ABSTRACT

Hemoglobin (Hb) is well protected inside the red blood cells (RBCs). Upon hemolysis and when free in circulation, Hb can be involved in a range of radical generating reactions and may thereby attack several different biomolecules. In this study, we have examined the potential damaging effects of cell-free Hb on plasmid DNA (pDNA). Hb induced cleavage of supercoiled pDNA (sc pDNA) which was proportional to the concentration of Hb applied. Almost 70% of sc pDNA was converted to open circular or linear DNA using 10 μM of Hb in 12 h. Hb can be present in several different forms. The oxy (HbO_2) and met forms are most reactive, while the carboxy-protein shows only low hydrolytic activity. Hemoglobin A (HbA) could easily induce complete pDNA cleavage while fetal hemoglobin (HbF) was three-fold less reactive. By inserting, a redox active cysteine residue on the surface of the alpha chain of HbF by site-directed mutagenesis, the DNA cleavage reaction was enhanced by 82%. Reactive oxygen species were not directly involved in the reaction since addition of superoxide dismutase and catalase did not prevent pDNA cleavage. The reactivity of Hb with pDNA can rather be associated with the formation of protein based radicals.

1. Introduction

Hemoglobin (Hb) is a tetrameric protein, composed of two alpha (141 residues) and two beta (146 residues) chains, forming an $\alpha_2\beta_2$ heterotetramer of 64 kDa. Each subunit harbors a heme in the center pocket responsible for its oxygen binding capability [1]. Although often viewed solely as an oxygen transporting protein, Hb has a very rich chemistry related to the reactivity of the iron atom in the heme groups. Hb is thus involved in several redox reactions and shows e.g. peroxidase-like activity, in which ferric or metHb (Fe3+) can react with hydrogen peroxide (H_2O_2) to form a potent oxidant ferryl Hb (Fe4+) along with protein-based radicals. These oxidized forms of Hb are in turn highly reactive and are involved in oxidative damages to a variety of biological molecules [2,3]. Modifications of particularly lipids and protein side chains caused by Hb have been examined in detail. Moreover, Hb can bind and react with nitric oxide (NO) to form nitrate and ferric heme. NO depletion through these reactions leads to vasoconstriction and platelet aggregation [4]. Hb is normally encapsulated in the protective environment of the erythrocytes, but when released upon hemolysis its redox reactions may cause serious damages to surrounding cells and tissues. This becomes especially pronounced at various hematological disorders or at blood transfusion events [5–7], when substantial amounts of cell-free or acellular Hb are released into the circulatory system. To prevent these harmful reactions, several defence proteins have evolved. The plasma protein haptoglobin (Hp), binds cell-free Hb rapidly and almost irreversibly, and transports the complex Hp-Hb to the CD163 receptors mainly located on the Kuppfer cells of the liver for enzymatic degradation and reuse [8,9]. Hemopexin and alpha-1-microglobulin are also involved in the protective system against toxic acellular Hb [10,11]. Cell-free Hb may thus insert a substantial burden to our body at a range of conditions and the use of Hb protective and degradative proteins have been proposed to soon also find clinical practice [12].

Despite its clinical manifestations, only limited knowledge of the interactions between Hb and nucleic acids are available. Hb can bind to calf thymus DNA and the protein also carries an endonuclease-like activity which has been examined by the conversion of supercoiled plasmid DNA (sc pDNA) to nicked circular DNA in the presence of H_2O_2 [13]. Similarly, myoglobin (Mb) showed a distinct pathway for cleavage of DNA upon chemically induced reductions in presence of oxygen or when Mb was present in its met form [14,15]. Hb can also induce DNA damages directly at the cellular level by rapid uptake and

Abbreviations: Hb, hemoglobin; metHb, ferric hemoglobin (Fe3+), HbA, adult hemoglobin; pDNA, plasmid DNA; sc pDNA, supercoiled plasmid DNA; ocDNA, open circular plasmid DNA; LDNA, linear plasmid DNA, CO, carbon monoxide; KCN, potassium cyanide; HbF, fetal hemoglobin; Hp, haptoglobin; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide

* Corresponding author.
E-mail address: leif.bulow@tbiokem.lth.se (L. Bulow).

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cleavage of available nucleic acids. This has been demonstrated for primary colon cells [16], leukocytes [17] and lymphocytes [18]. The use of the comet assay has been instrumental for quantifying the genotoxic effects of Hb, which occur rapidly and already at low concentrations.

In the human population several different Hb variants have been identified [19]. In this study, wild-type and mutant Hb molecules have therefore been examined to characterize their reactivity to nucleic acids. For this we have studied plasmid DNA (pDNA) cleavage by Hb. Purified pUC18 plasmid was incubated with various Hb samples, adult and fetal Hb, i.e. HbA and HbF, respectively, at different experimental conditions. Hemin (free heme in the met form) was included in the study to evaluate the effects of globin polypeptides and heme separately. It was observed that metHb interacts with pDNA and exhibit high endonuclease activity, responsible for conversion of sc pDNA into open circular DNA (ocDNA) or linear DNA (LDNA). Interestingly, HbF, which shares the same alpha chains as HbA, but has unique gamma chains, exhibited no or only low measurable reactivity against nucleic acids. This may have several clinical implications for treatment of a range of hematological conditions, like sickle cell anemia (SCA) and different forms of thalassemia.

2. Material and methods

2.1. Plasmid purification

Plasmid was prepared and isolated as described in our previous work [20]. The *E. coli* strain TG1 (GE Healthcare, Uppsala, Sweden) was used as host in all experiments for production of plasmid pUC18 of 2686 base pairs (GE Healthcare, Uppsala, Sweden). Cells were grown in Luria-Bertani (LB) media at 37 °C overnight, harvested by centrifugation and plasmid DNA was isolated using a dedicated purification kit (NucleoSpin Plasmid, MACHEREY-NAGEL, Germany). The DNA was eluted with 20 mM sodium phosphate buffer pH 7.2 and purified plasmid was stored at −80 °C until further use. The concentration of pDNA was determined by an Implen Nanophotometer (Labvision AB, Sweden).

2.2. Hb preparation

Recombinant HbA was constructed, expressed and purified as described earlier [3]. In brief, the alpha and beta chains of Hb were cloned in the pETDuet-1 vector and transformed into *E. coli* strain BL21 (DE3). Transformed cells were grown in Terrific Broth (TB) media with vigorous shaking (150 rpm) in baffled 2 L flasks at 37 °C until OD_{620}≥2. The cultures were then induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG, Saveen Werner AB, Sweden) and supplemented with 0.3 mM δ-aminolevulinic acid (Sigma Aldrich), and allowed to grow overnight at 22 °C with reduced aeration (60 rpm). Cells were collected and sonicated in 10 mM sodium phosphate buffer pH 6.0. Protein was purified using a weak (CM Sepharose, GE Healthcare, Sweden) followed by strong (Q Sepharose HP, GE Healthcare, Sweden) ion exchange chromatography. The purified recombinant Hb samples were identical with the native protein and stored at −80 °C until further use. To keep the Hb samples in a stable form, carbon monoxide (CO) gas was bubbled through all solutions used at induction and each step of the purification.

The ferric form of Hb (metHb) was obtained by incubating Hb with 10 mM excess of potassium ferricyanide (Sigma Aldrich). This solution was passed through a gel filtration column Sephadex G-25 (5×0.5 cm, GE Healthcare, Sweden) to remove excess of ferricyanide. The concentration of Hb was determined by reducing an aliquot of the ferric Hb with sodium dithionite (Sigma Aldrich) to the deoxy form (430 nm=133 mM^{-1} cm^{-1}). The Hb concentration used in all experiments is given on the basis of molar heme. To obtain the oxy form of Hb, the CO-Hb was exposed to a continuous stream of oxygen gas in presence of light. All the experiments were carried out in 20 mM sodium phosphate buffer pH 7.2 at 37 °C using the ferric form of Hb or as otherwise specified.

2.3. DNA cleavage assay

The pDNA (25 μg/ml) was incubated with different concentrations (1–100 μM) of the ferric Hb at 37 °C using PCR tubes (total reaction volume of 20 μl). Sample was removed after every 2 h and analysed by agarose gel (1%) electrophoresis carried out for 60 min at 100 V using TAE buffer (Tris-acetate-EDTA, pH 8.0). A control sample was included in all experiments containing only pDNA. The different forms of DNA (supercoiled, open circular and linear) were quantified by densitometric analysis using the 'Quantity one' software from Biorad. To evaluate the temperature dependent degradation of pDNA, Hb (20 μM) was incubated with pDNA at different temperatures (10–40 °C). The DNA damage effects were also evaluated in different concentrations of sodium phosphate buffer (20, 50 and 100 mM, pH 7.2). Similarly, oxy and CO adducts of Hb were examined. The absorption spectra of Hb (5 μM) with or without pDNA was taken every 30 min at 37 °C for 12 h using a Cary60 UV–Vis spectrophotometer (Agilent Technologies). All data were analysed from minimum three independent experiments.

2.4. Preparation of HbF mutants

Site-directed mutagenesis was employed for removing a cysteine residue in the gamma chain (γ-C93A) close to the heme group. Similarly, an alanine residue located on the surface of the alpha chain was substituted to cysteine (α-A19C). Primers used were purchased from integrated DNA technologies (IDT, Germany). Forward sequence of primers employed are as follows (reverse primers were complementary to forward primers): bold sequence represents mutation, 5′-GGGGTAAAGTTGGT**TGC**CATGCCGGTGAA-3′ (α-A19C) and 5′-GAGTGAACTGCAC**GCC**GATAAACTGCAC −3′ (γ-C93A). A double mutant was prepared using similar primers. After PCR, the methylated DNA was removed using DpnI (Thermo Scientific) digestion. These mixtures were transformed into *E. coli* strain BL21 (DE3). Sequences of the transformed clones were confirmed (GATC Biotech, Germany). The expression conditions were slightly different than for HbA. In brief, the starter culture was inoculated into 500 ml TB media and was induced immediately. This culture was allowed to grow overnight at 30 °C and 150 rpm. These mutants and wildtype HbF variants were purified as described elsewhere [21]. HbF and HbF mutants (25 μM) were incubated with supercoiled plasmid pUC 18 (10 μg/ml) at 37 °C. Sample were removed after 1, 2 and 3 h respectively, and analysed by agarose gel electrophoresis.

2.5. Inhibition studies of Hb reactivity

A potassium cyanide (KCN) solution (100 μM, Sigma Aldrich) was mixed with Hb (50 μM) prior to the experiment for 1 h to allow complete complexation of the heme iron to prevent any oxidation reaction. The complex was subsequently incubated with pDNA (25 μg/ml) at 37 °C for 12 h. In another set of experiment, Hb samples were incubated together with pDNA containing sodium chloride (100 mM and 500 mM). Similarly, metHb (20 μM) and pDNA was incubated with superoxide dismutase (SOD, 20 units, Sigma Aldrich), catalase (20 units, Roche), ascorbate (20 μM, Sigma Aldrich) and dimethyl sulfoxide (DMSO, 100 μM).

2.6. Protection by haptoglobin

A haptoglobin (Hp) sample containing primarily dimers (Hp 1-1) and to a lesser extent polymer (Hp 2-1, and Hp 2-2) was kindly provided by Bio Products Laboratory (BPL, Hertfordshire, UK). Hp: Hb in ratios (1:1.25, 1:0.625, 1:0.25, 1:0.125) were incubated for 10 min.