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# Aggregation of adult and fetal hemoglobin by ingested nitrate anions

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## ABSTRACT

The ingested nitrates sourced from tap water, food, chemicals and pharmaceuticals are converted to nitrites in the body surfaces by bacteria and then, the nitrite ions can lead the structural changing in hemoglobin. In the present work, aggregation of the purified hemoglobin in adult (HbA) and in fetus or newborn (HbF) in the presence of nitrite ions were studied. Hemoglobin aggregation was performed chemically in the presence of 10 mg/l nitrite ions and examined by UV-Vis spectrophotometer at 360 nm wavelength. The extrinsic fluorimetric measurements indicated that repulsive electrostatic interaction between nitrite anions and negative charged groups of both types of HbA and HbF molecules leads to expose the hydrophobic patch of the protein molecules. Moreover, the α-helix to β-strand transition in both types of hemoglobins shown by circular dichroism support aggregation process among this protein. However, at natural pH, the protonated amino group of Gly in HbF tends to bind to nitrite anions more than the unprotonated forms of Val residue in HbA. The drastic slop of aggregation plot and shorter lag time of HbF relative to HbA demonstrated more aggregation of former protein.

**Keywords:** adult hemoglobin, aggregation, nitrate ions, fetal hemoglobin, nitrite ions.

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## Introduction

Nowadays, the protein aggregation has become a serious problem for human health. It causes illnesses like Alzheimer's disease (1, 2), Parkinson's disease (3, 4) and monoclonal immunoglobulin amyloidosis (5, 6). Recent studies suggest that aggregation is much more likely to arise from specific partially folded intermediates. The essential factors which are effective on protein aggregation are protein sequence, pH, temperature, ionic strength, protein concentration, molecular chaperone, co-solutes or denaturants such as urea, and chaotropes or kosmotropes including osmolytes and ligands that interact selectively with protein conformation or protein aggregated particles (7). The aggregates formed during protein folding process have been assumed due to hydrophobic aggregation of the unfolded or denatured states, whereas amyloid fibrils and other extracellular aggregates have been assumed to arise from native-like conformations in a process analogous to the polymerization of hemoglobin S (8). Protein aggregation is an entropy driven process (9) which could be distinguished as: reversible association of the native monomer, aggregation of conformationally-altered monomer, aggregation of chemically-modified product, nucleation-controlled aggregation, and surface-induced aggregation (10).

Hemoglobin (Hb) is a physiologically significant globular protein (11) with a molecular weight of 64.5 kDa. Adult hemoglobin (HbA) consists four globin chains including two α-chains and two β-chains (12, 13). Each of globin chains contains a heme prosthetic group. The heme group is located in position by interactions with the histidine side chain of globin (14). Accounting for approximately 80% of total hemoglobin in newborns and the main component during fetal life and at birth is fetal hemoglobin (HbF). After birth, HbF synthesis is swiftly declined and HbF is gradually substituted by HbA in the blood. HbF is organized by two α- and two γ-globin chains consisting of 141 and 146 amino acid residues, respectively, while α-chains are the same as those contained in HbA (15). Functionally, HbF differs mostly from HbA because it has somewhat higher oxygen affinity. This characteristic makes the delivery of oxygen through placenta easier, giving fetus better access to oxygen from the mother's bloodstream (16). HbA and HbF have the same molecular weight but they differ in solubility, amino acid composition, electrophoretic mobility, spreading on liquid surfaces and resistance to denaturation by alkali and in immunological attributes. HbA and HbF are identical in heme prostetic group but they differ in the arrangement and composition of the globins and perhaps in the linkage between globin and heme (17). By oxidizing the iron atom in hemoglobin from ferrous state ($Fe^{2+}$) to ferric state ($Fe^{3+}$), a met-hemoglobin (MetHb) is produced (18, 19).

Ingested nitrates are converted to nitrites by facultative anaerobic bacteria, commensal in the gastrointestinal tract and saliva and on body surfaces (20). Nitrite ions can lead conversion of oxy/deoxy Hb to MetHb (21). Nitrates and nitrites sources include water, food, chemicals, and pharmaceuticals (22, 23). Blue baby syndrome is consequence of elevated levels of nitrite in blood of infant due to the infantile met-hemoglobinemia (24). However, the mechanism of reaction between oxy-hemoglobin (OxyHb) and nitrite has not yet been fully clarified (25). In some studies, the relationship between nitrate levels in drinking water and the risk of childhood type 1 diabetes (26), pancreatic cancer (27),

and cardiovascular disease was considered (28). In the present study, the process of aggregation of HbA and HbF caused by nitrite ions was investigated.

## Materials and Methods

Potassium dihydrogen phosphate ($KH_2PO_4$) and dipotassium hydrogen phosphate ($K_2HPO_4$) were purchased from Merk (Germany). 8-Anilino-1-naphthalenesulfonic acid (ANS) and other chemicals were obtained from Sigma-Aldrich. The sodium nitrite solutions were prepared from sodium nitrite salt ($NaNO_2$) (Sigma, USA) in phosphate buffer solution (PBS, 50 mM, pH 7.4). Then, they were diluted to 10 mg/l in deionized water. Adult OxyHb was extracted from human blood and HbF was extracted from umbilical cord blood of newborn and purified according to the literature (29). HbF is more stable to alkaline conditions relative to HbA (30). Therefore, HbF was purified by adding NaOH (3 M), and adjusting the pH to 13. Then HbF was dialyzed three times against PBS. The purified HbA and HbF aliquots were frozen in liquid nitrogen and stored at -70°C (31).

According to U.S. Environmental Protection Agency, the conversion rate of nitrate reduction to nitrite is 10% in infants (32). Therefore, drinking water such as the tap water in Tehran (measured on May 2010) which was containing 100 mg/l nitrate ion could be converted to 10 mg/l nitrite ion in infant's body. In addition, the ingested nitrate in adult is rapidly reduced to nitrite by bacteria in the mouth. Although much of the nitrate ingested (65-70%) is excreted in the urine, but part of it (25%) converts to nitrite and enters the systemic circulation (33). Therefore, in order to study the effect of ingested nitrate anions on the aggregation of adult or fetal HB, $NaNO_2$ (10 mg/l) was exposed to either HbA or HbF. For this purpose, 50 µL of the salt was added to 200 µL hemoglobins and the samples were incubated at 37°C for 7 days in aseptic conditions. The interaction between nitrite and HbA and HbF samples were investigated in different incubation times and finally seven days incubation was selected as optimum time (data not shown). The aggregation of HbA and HbF in the presence of nitrite ions was also investigated by measuring absorbance at 360 nm in 37˚C using a cary 100 UV-Vis Spectrophotometer (29).

Dynamic light scattering (DLS) was also applied to monitor the molecular size changes of proteins upon denaturation due to the nitrite ion exposure (27). DLS (Zeta Pluse, Brookhaven, USA) was used for molecular size changes of HbA and HbF samples at concentration of 0.3 µM in PBS 50 mM (pH 7.4).

The secondary structure of HbA and HbF in the presence and absence of nitrite ions was investigated by circular dichroism spectrophotometer (CD), Aviv 215 (USA), in a 0.1 cm cuvette at 190-260 nm. The CD spectra were analyzed by CDNN software. All CD measurements were made at 25˚C. The baseline spectrum of the buffer was subtracted from each spectrum, and resultant values were expressed in mean residue ellipticity [θ] (deg $cm^2$ $dmol^{-1}$) based on the mean amino acid residue weight of 110 Da (MRW). The mean residue ellipticity was defined as $[\theta]_\lambda = (100MRW\theta/c\times l)$, where θ is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/ml and l is the length of the cell (cm) (28).

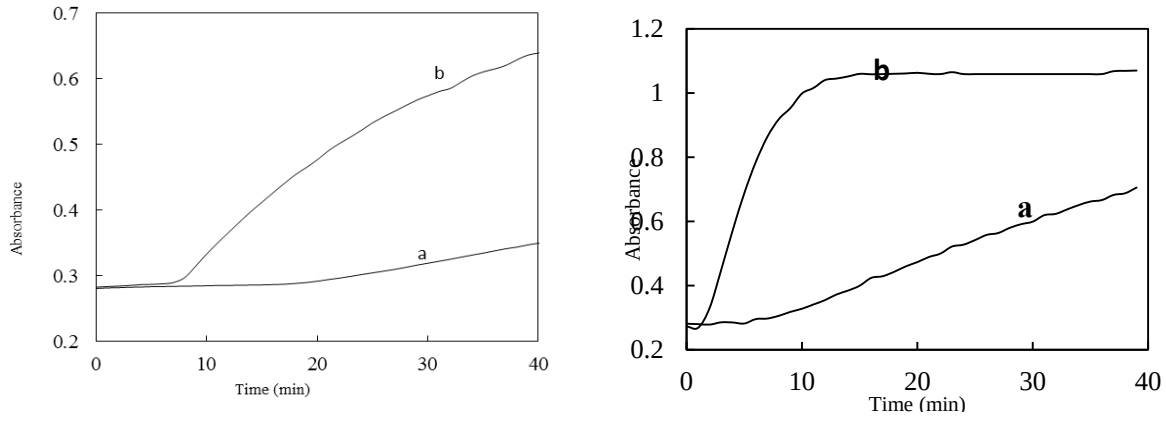
The change in fluorescence of ANS (300 µM) as a hydrophobic probe upon binding to HbA or HbF (3 µM) in the presence and absence of nitrite ions was measured. ANS was incubated with protein solution for 10

min at 37˚C. A spectrofluorimeter (Carry Eclips model, Varian, Australia) was used to record the fluorescence spectra of ANS-HbA and ANS-HbF in the presence and absence of 10 mg/l nitrite ions. The excitation wavelength used was 385 nm and excitation and emission window diagonal were 5 nm and 10 nm, respectively (29).

## Results and Discussion

*UV-Visible Spectroscopy*

A traditional and simple instrumental method in monitoring protein aggregation is the turbidimetric method by measuring the optical density of the sample based on light scattering in the near UV or visible region, where proteins have negligible absorption (29). Polymerization of protein molecules due to their physical instability may lead to aggregation. Thermal denaturation which is particularly widely used in protein stability studies is often irreversible (30). Hemoglobins aggregation was studied by measuring the change in absorbance at 360 nm and 37°C both at normal state and in the presence of nitrite ions in solution. In Figure 1A the absorbance of HbA in the absence and presence of nitrite ions is compared. As seen, no significant change in absorbance was observed for HbA in its native form but in the presence of nitrite ions a drastic change started at 8th min and continued till 40$^{th}$ min. The remarkable slop of absorbance at 360 nm and about 8 min lag time indicates the formation of HbA aggregation. The abridging the lag time and enhancement of the slope of aggregation curves suggest that the hemoglobin molecules is suffered the aggregation phenomenon (28).




**A)** **B)**

**Figure 1. UV-Vis spectra of HbA (A) and HbF (B) in the absence (a) and presence (b) of 10 mg/l nitrite ions at 360 nm and 37˚C**

In Figure 1B the absorbance of HbF in the absence and presence of nitrite ions is presented. As seen, the absorbance of HbF suffers a significant change in the presence of nitrite ions. Comparing to HbA, the lag time has a noticeable decrease so that a drastic change started at first min and reaches to a plateau at 10th min. In the presence of nitrite ions the lag time for aggregation of HbF in comparison with HbA was shortened. This was associated with the steep of the aggregation slope and maximum absorbance for HbF in the presence of nitrite ions relative to HbA.

It seems that the primary effect of introducing anions to juxtaposed positive

charges on protein surface is reducing electrostatic repulsion of the protein charge groups. But, longer effects may include electrostatic interaction of β-143 His and the $NH_2$-terminal group of β-1 Val (34). The N-terminal residue of the β-subunit in HbA is Val, which has a pKa of 6.6, but the corresponding Gly on the γ-subunit of HbF has a much higher pKa of 8.1 (35). Because protonated amino group of Gly in HbF tends more bind to anions like nitrite ions than the unprotonated forms of Val residue, HbA binds less nitrite anions. The more accumulation of negative charges of nitrite ions around HbF leads to increase repulsive interactions with negative charges of fetal hemoglobin surface. Then, HbF may bury the electrostatic sites and exposes the hydrophobic patch to surface of molecule to reduce repulsive interactions. Consequently, hydrophobic areas increasing can rises aggregation possibility.

Thermostability of hemoglobin in the presence of nitrite ions was controlled by preparing the transition midpoint ($T_m$). The results showed that $T_m$ decreased in the presence of the nitrite ions suggested that the thermal stability of protein was also diminished. $T_m$ of HbA in native form was 64˚C and in the presence of 10 mg/l nitrite decreased to 61˚C. Also, $T_m$ of HbF in native form was 60˚C which declined to 56˚C after incubation with 10 mg/l nitrite ion. These results indicated that nitrite ions affected on the thermal stability of HbA and HbF.

*Light scattering of protein aggregation*

There are numerous investigations of the aggregation kinetics for different proteins (9). However, it is obvious that more reliable information about the mechanism of protein aggregation could be obtained using the methods that allow sizing the aggregated proteins. On-line analysis of the kinetics of protein aggregation can be carried out using DLS. Colloidal particles or macromolecules suspended in fluid undergo Brownian motion. This motion causes fluctuations in the local concentration of the particles resulting in local non-homogeneities of the refractive index. This in turn leads to the light scattering phenomenon (36). In the presence of ions, the hydrophobic interactions between hydrophobic sites of hemoglobin exposed to surface of protein lead to associate of hemoglobin molecules to each other. Therefore, by addition of the nitrite ions, the particle size of hemoglobins is increased. As seen in Figure 2, increasing the particle size in HbF is more impressive than that of HbA indicating that HbF aggregation is more tangible than that of HbA as a result of nitrite ions.

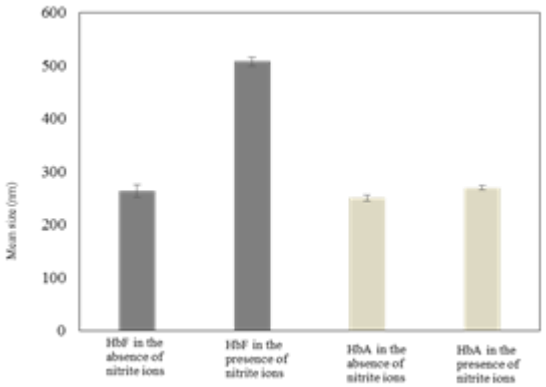
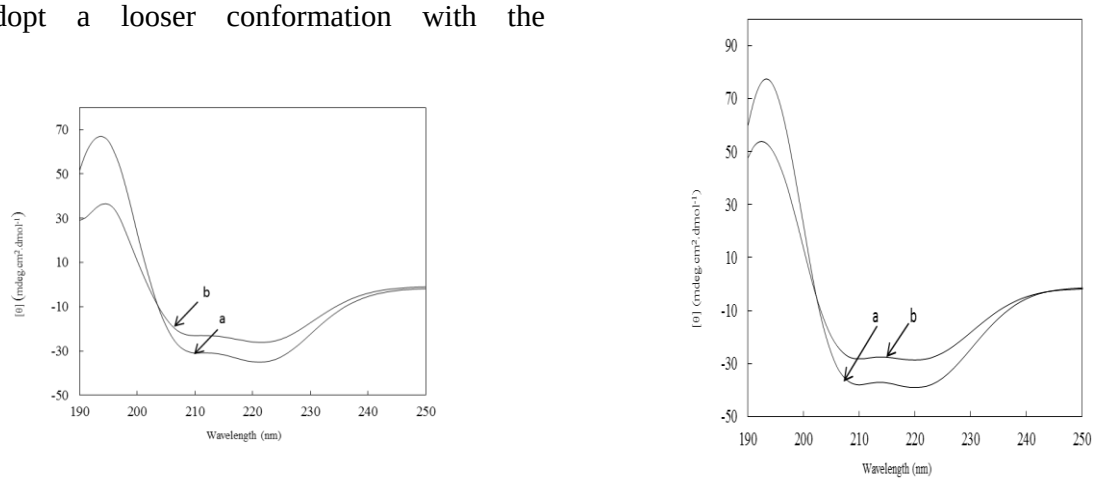



**Figure 2. Mean size of HbA and HbF in the presence and absence of nitrite ions**

*The secondary structural changes*

Changes in far-UV CD signals can be used to monitor the loose of structure during unfolding (37). The secondary structural changes of HbA and HbF were shown in the far-UV CD region (Fig. 3A and 3B). As seen, the far-UV CD spectra of Hb exhibited two negative bands in the ultraviolet region at 208 and 222 nm, which indicates the α-helical characteristic of the Hb (38). $NO_2^-$ may induce conformational change in hemoglobin

via electrostatic interaction between negative charge ions and the negative charges of residues in the Hb structure. Therefore, the molar ellipticity change in the wavelength range from 190 to 260 nm was used to monitor the secondary structure change of HbA and HbF, in the presence and absence of nitrite ions. The decrease in α-helix content suggests that the Hb molecules probably adopt a looser conformation with the extended polypeptide structures (38). α-helix to β-sheet transition is fundamental feature that in associating with misfolding of proteins leads to protein aggregation (39). From these results, it is apparent that interaction of nitrite ions with Hb causes a conformational change of the protein with decreasing α-helix and increasing β-structure and leads to α→β transition (40).




**A)** **B)**

**Figure 3. The far-UV CD spectra of HbA (A) and HbF (B). The spectra were obtained for HbA and HbF at concentration of 3 µM in the absence (a) and presence (b) of 10 mg/l nitrite ions at 37˚C**

The approximate percentage of each secondary structure can be estimated by analyzing the protein CD spectra in far-UV region. In this work, the secondary structural elements for HbA and HbF are calculated from CD data at pH 7.4 using CDNN program. According to Table 1, native HbA possesses 82% helix, 10% beta-structure and 8% random coil. While, in the presence of 10 mg/l nitrite the helical content significantly decreased to 64% whereas the beta-structures and random coil increased to 19% and 17%, respectively. In addition, in the presence of 10 mg/l nitrite, the α-helix content of HbF decreased about 15%, while the beta-structure and random coil increased 16% and 12%, respectively. Decreasing the α-helix content of HbA and HbF and increasing the random structure indicates losing the secondary structure.

**Table 1. The secondary structure of HbA and HbF in the presence and absence of nitrite ions. Data were extracted from Figure 3 using CDNN program.**

| Secondary structure (%) | α helix (%) | β sheet (%) | Random coil (%) |
|---|---|---|---|
| HbA in the absence of ions | 82 | 10 | 8 |
| HbA in the presence of ions | 64 | 19 | 17 |
| HbF in the absence of ions | 87 | 9 | 4 |
| HbF in the presence of ion | 72 | 16 | 12 |

The conformational transition probably results in the exposure of the hydrophobic cavities and a perturbation of microenvironments around the aromatic amino acid residues (41). On the other hand, nitrite ions decrease the helical content in both HbA and HbF at 37˚C. However, the repulsive interactions between nitrite ions and negative charge in HbF are stronger than those in HbA. Therefore, replacing the hydrophobic patch with electrostatic area in HbF should be more significant that in HbA. This conclusion is compatible with those obtained by spectroscopy, too (Fig. 2).

*Conformational changes in HbA and HbF*

In order to characterize the exposed hydrophobic surfaces of both types of hemoglobins due to the presence of nitrite ions, the fluorescence spectra of ANS were measured at different conditions. Since in the surface of the native HbA and HbF some hydrophobic sites are available for ANS binding, an increase in ANS fluorescence was observed after incubation of HbA and HbF with nitrite ions (Fig. 4A and 4B). In fact, repulsive electrostatic interaction between negative charges of $NO_2^-$ ions and negative charged groups of the HbA and HbF presumably cause Hb molecules to unfold and expose hydrophobic sites to the aqueous environment. The increase in the hydrophobic patches at hemoglobins surrounding which detected by ANS fluorescence indicates the growth of macromolecular aggregates principally through hydrophobic interactions [42].

Accumulating unfolded hemoglobin molecules led to interact via exposed hydrophobic sites and form large aggregates. However, the overall structural changes in the presence of nitrite ions lead to exposure of more hydrophobic surfaces and significant increase in ANS fluorescence intensity of HbF relative to HbA.

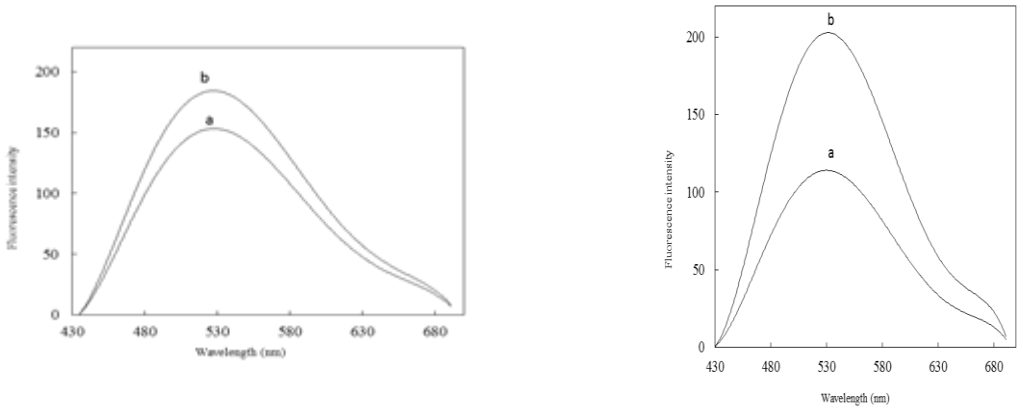



**Figure 4. ANS fluorescence spectra of HbA (A) and HbF (B). The spectra were obtained for HbA and HbF at concentration of 3 µM in the absence (a) and presence (b) of 10 mg/l nitrite ions at 37˚C**

## Conclusion

The experimental results obtained in the present work revealed that in the presence of nitrite anions (>10 mg/l), both HbA and HbF tend to aggregate. The reason for such an aggregation could be due to the secondary structure loose during the α-helix to β-sheet transition. This process in turn, exposes the hydrophobic patch of HbA and HbF molecules to nitrite anions in which the repulsive electrostatic interaction between negative charged groups on HbA and HbF surface and nitrite anions are strengthened. However at neutral pH, the protonated amino

group of Gly in HbF tends to bind to nitrite anions more than the unprotonated forms of Val residue in HbA. Therefore, it seems that in the presence of nitrite anions HbF molecules are more talent for aggregation than HbA molecules.

## Acknowledgment

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