Interaction of hemin with quadruplex DNA

Maryam Ghahremani Nasab¹ · Leila Hassani¹ ·
Sara Mohammadi Nejad¹ · Davood Norouzi²

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Abstract A DNA enzyme with peroxidase activity is a G-quadruplex-based DNAzyme formed by hemin and G-quadruplex DNA. Activity of peroxide DNAzymes can be influenced by the structure of quadruplex DNA. In this investigation, the interaction of hemin with T30695 G-quadruplex DNA is evaluated. Molecular dynamic simulation indicates that the binding mode of hemin to G-quadruplex DNA is end-stacking, which is consistent with absorption spectroscopy. Based on fluorescence spectroscopy, hemin ejects thiazole orange from bases of four-strand DNA. Circular dichroism spectra showed that no alteration occurs in this type of DNA structure.

Keywords DNAzyme · Peroxidase · Quadruplex · Hemin · Interaction

1 Introduction

In nature, DNA stores genetic information and exhibits no enzymatic activity, although in recent years catalytic properties of DNA, such as the cleavage of DNA or RNA, DNA modification, ligation, and metalation of porphyrin rings are well known. DNAzymes are not found *in vivo* and all of them are artificially prepared [1]. There are some remarkable advantages in using DNA instead of RNA or protein, which could be leveraged to the practical use of DNAzymes. For example, DNA is much more stable than RNA and protein against hydrolysis and preparing DNA is much more affordable than protein [2].

The DNA enzyme with peroxidase activity is a well-known DNAzyme. It is a G-quadruplex-based DNAzyme formed by hemin and G-quadruplex DNA [3, 4]. Heme is a universal metabolic cofactor and contributes in a variety of cellular roles, counting electron

✉ Leila Hassani
hasani@iasbs.ac.ir

¹ Department of Biological Sciences, Institute for Advanced Studies in Basic Sciences (IASBS), Zanjan 45195-1159, Iran

² Laboratory of Cell Biology, National Cancer Institute NIH, Bethesda, MD 20892, USA

removal, the carriage and detecting of diatomic gases, and several types of oxidative catalysis. Its molecular formula is $C_{34}H_{32}ClFeN_4O_4$ with a complex circular organic structure, known as protoporphyrin, containing a ferric iron ion [5]. G-quadruplexes are four-stranded nucleic acid structures formed by hydrogen bonding of four guanine bases in G-tetrads. They can form intra- and inter-molecular structures stabilized by three or more G-tetrads [6].

It has been recognized that many aptamers and DNAzymes, such as peroxidase DNAzymes, can adopt quadruplex structures. In peroxidase DNAzymes, noncovalent attachment of quadruplex DNA to hemin leads to improved peroxidase activity of hemin and facilitates a redox reaction between the target molecule and hydrogen peroxide [1]. DNAzymes with peroxidase activity have recently attracted great interest in medicine, biology, and material sciences. Some applications of this class of DNAzymes include detection of specific DNA sequences, measurement of telomerase activity, determination of heavy metals, and detection of small molecules and proteins such as cocaine and thrombin [1, 7–10].

It is well known that the activity of peroxide DNAzymes can be influenced by the structure of quadruplex DNA [7, 11]. G-quadruplexes can form various conformations depending on the oligonucleotide sequence and concentration, buffer conditions, temperature, the presence of monovalent cations such as K^+ and Na^+ , and length of loops [6]. Tetramolecular structures with parallel strands and unimolecular antiparallel quadruplexes show low peroxidase activity, while parallel or mixed intramolecular quadruplexes exhibit the highest activity. It was suggested that a parallel structure is more favorable for binding of hemin to quadruplex DNA [1, 7, 11]. The structure of the G-quadruplex influences its binding to hemin and therefore the interaction between the hemin group and G-quadruplexes DNA is an important factor for catalytic activity of the peroxidase DNAzymes. Accordingly, the major aim of this study is to investigate the binding of hemin to G-quadruplex DNA. To do this, T30695 was selected as G-quadruplex DNA. T30695 is a G-quadruplex DNA aptamer that binds with high affinity to two different proteins, namely HIV 1 integrase (HIV-IN) and interleukin-6 receptor (IL-6R) [12–14].

The interaction of K^+ and Na^+ forms of T30695 with hemin was evaluated by spectroscopic techniques and molecular dynamic simulation. The catalytic activity of the G-quadruplex-based DNAzyme was also measured by fluorescence spectroscopy.

2 Materials and methods

2.1 Preparation of G-quadruplex DNA

The oligonucleotide T30695 (GGGTGGGTGGGTGGGT) was prepared by dissolving in HEPES buffer 20 mM containing KCl 150 mM or NaCl 150 mM and EDTA 0.1 mM, pH 7.2 and then heated to 90 °C for 5 min, cooled to room temperature, and incubated at 4 °C overnight.

2.2 UV–Vis absorption spectroscopy

UV–Vis spectroscopic titrations were performed using an Analytic Jena 2000 spectrophotometer at room temperature. The quartz cells path lengths were 1 cm. UV–Vis absorption titrations were done by addition of DNA 200 μ M stock solution to a cuvette containing 800 μ l of hemin 10 μ M in HEPES buffer 20 mM containing KCl 150 mM or NaCl 150 mM.

The percentage of hypochromicity was calculated using:

$$\text{Hypochromicity (\%)} = \frac{|\varepsilon_f - \varepsilon_b|}{\varepsilon_f} \times 100$$

where ε_f and ε_b correspond to the extinction coefficient of the free and bound forms of the complex.

2.3 Fluorescence spectroscopy

Fluorescent spectra were taken on a Perkin-Elmer (Varian, Australia) spectrofluorometer. The measurements were carried out using a 1-cm quartz cell and bandwidths of 5 and 10 nm for excitation and emission monochromators, respectively. Thiazole orange was excited at 501 nm. The molar ratio of thiazole orange (1 μM) to the oligonucleotide (1 μM) was 1:1.

2.4 Circular dichroism measurements

Circular dichroism (CD) measurements were performed on an Aviv Model 215 Circular dichroism Spectrometer (Lakewood, NJ, USA) at 25 °C using quartz cells with a path length of 1.0 mm. Two oligonucleotide samples were prepared. The first sample was prepared in HEPES buffer 20 mM containing EDTA 0.1 mM, triton X-100 0.025%, KCl 150 mM, pH 8. In the second sample, KCl 150 mM was replaced with NaCl 150 mM. The concentration of the oligonucleotide and hemin in both samples was 10 μM . The molar ellipticity was determined as $[\theta] = (\theta \times 100 \text{ MRW})/cl$, where c is the oligonucleotide concentration in mg/ml, l is the light path length in centimeters, MRW is the mean residue weight of the oligonucleotide and θ is the measured ellipticity in degrees [15].

2.5 Measurement of DNAzyme activity

DNAzyme activity was measured by fluorescence spectrofluorometer. The experiments were performed at an excitation wavelength of 330 nm and the emission spectra were recorded in the range of 350–550 nm. The measurements were performed using a 1-cm quartz cell and bandwidths of 5 and 10 nm, respectively, for excitation and emission monochromators. In this study, measurements were made with 0.5 μM DNAzyme, 0.25 μM hemin, 0.5 mM luminol, and 5 mM H_2O_2 . Briefly, 2 μl of DNAzyme solution, 250 μl of reaction buffer, and 250 μl of 2 mM luminol solution were added to the cuvette serially. Then, 250 μl of 20 mM H_2O_2 stock solution was added quickly into the cuvette and emission spectra were recorded. Two samples were prepared; sample 1 was prepared in HEPES buffer 20 mM containing EDTA 0.1 mM, DMSO 1%, triton X-100 0.025%, KCl 150 mM, pH 8; in sample 2, KCl 150 mM was replaced with NaCl 150 mM pH [16–19].

2.6 Molecular modeling simulations

Simulation was performed using the AMBER molecular dynamics package version 10. The NMR structure of the G-quadruplex (GGGTGGGTGGGTGGGT) was taken from a protein data bank (PDB ID: 2LK7). The molecules were placed in a periodic rectangular box and an implicit model

was used to model the solvent. Potassium ions were added to the system to neutralize the electric charge of the molecules. We employed FF99SB and FFBsc0 force fields, respectively, for hemin and DNA molecules. The simulation time step was 1 fs. Energy minimization and molecular dynamics were performed by using a sander module. Minimization was performed in order to remove improper interaction between atoms. Before the main molecular dynamics step, the system was equilibrated for 1000 steps; 500 steps with the steepest descent method followed by 500 steps with the conjugate gradient method. In this procedure, all atoms involving hydrogen are constrained using the SHAKE algorithm. In the following step, the main MD simulation was run for 50 ns. The temperature was kept at 300 K by using a Langevin thermostat under 1 atm pressure.

3 Results and discussion

3.1 Absorption spectroscopy

In this investigation, spectrophotometry, spectrofluorometry, and circular dichroism spectroscopy were used to evaluate the interaction between the G-quadruplex and hemin in the presence of KCl and NaCl. The absorption spectrum of hemin displayed an absorption peak around 396 nm in an aqueous buffer solution. Figure 1a and b display an alteration in the absorption spectra of hemin after titration with the G₄ DNA in presence of K⁺ and Na⁺ ions, respectively. As observed, the intensity of the solet band increases with addition of the G₄ DNA and then a red shift appears with an increase in the concentration of G₄. The values of red shift and hypochromicity (%) were also plotted against the concentration of hemin and are presented in Fig. 2. The maximum hyperchromicity of hemin is 52 and 46% in the presence of K⁺ and Na⁺, respectively, and the maximum bathochromic shift of both cases is about 10 nm. This result indicates that there is no remarkable difference between the absorption spectra of hemin due to the interaction with Na⁺ and K⁺ forms of the G-quadruplex. As is well known, intercalation of porphyrins between the base pairs of double helix DNA leads to about a 15-nm red shift and almost 35% hypochromicity of the porphyrins solet band. A small red shift (about 6–8 nm) and hypochromicity (10%) or even hyperchromicity of the solet band are observed due to outside binding without stacking of porphyrin to double helix DNA [20, 21]. These spectral characteristics have been determined for long pieces of duplex DNA but not for the quadruplex DNA. Obviously, for titrations of hemin with K⁺ and Na⁺ forms of the

Fig. 1 Absorption spectra of hemin titrated with K⁺ and Na⁺ forms of the quadruplex DNA

Fig. 2 Wavelength of maximum absorption, $\lambda_{\max}$ (a) and maximum absorption (b) of hemin titrated with K⁺ and Na⁺ forms of the quadruplex DNA

quadruplex, the hyperchromicity is large and the red shift is not exactly in the range of outside binding and intercalation but, the spectral characteristics resemble more the outside binding than intercalation. Although it is not possible to discriminate groove binding from end-stacking using absorption spectroscopy, we guess that one of the binding modes of hemin to the quadruplex DNA is end-stacking. End-stacking is an interaction mode that could occur on quadruplex DNA but not long pieces of duplex DNA.

Figure 1a and b show an isosbestic point at 422 nm that appeared after titration of G₄DNA on the hemin solution indicating equilibrium between free hemin in the solution and the hemin/G₄ DNA complex.

The binding constant (K_b) values were calculated by the following equation [22]:

$$\frac{[\text{DNA}]}{|\varepsilon_a - \varepsilon_f|} = \frac{[\text{DNA}]}{|\varepsilon_b - \varepsilon_f|} + \frac{1}{K_b (|\varepsilon_b - \varepsilon_f|)}$$

where [DNA] is the concentration of DNA in base pairs, ε_a , ε_f and ε_b correspond to $A_{\text{observed}}/[\text{the complex}]$, the extinction coefficient of the free complex and that of the complex in the fully bound form, respectively, and K_b is the intrinsic binding constant. A plot of $[\text{DNA}]/(|\varepsilon_a - \varepsilon_f|)$ versus [DNA] will have a slope of $1/(|\varepsilon_b - \varepsilon_f|)$ and an intercept equal to $1/(K_b(|\varepsilon_b - \varepsilon_f|))$. K_b is then given by the ratio of the slope to the intercept. The binding constant of interaction between hemin and Na⁺ and K⁺ forms of the quadruplex are $1.9 \times 10^{-5} \text{ M}^{-1}$ and $1.1 \times 10^{-5} \text{ M}^{-1}$, respectively. The result indicates that K_b for the Na⁺ form is larger than for the K⁺ form, indicating that the binding affinity of hemin to the Na⁺ form is stronger.

3.2 Fluorescence spectroscopy

Fluorescent intercalator displacement (FID) is a well-known assay based on the loss of fluorescence of a DNA-bound intercalator or minor-groove binder on displacement by a DNA-binding molecule. Thiazole orange (TO) is generally used as a DNA-bound intercalator. Initial studies have demonstrated that thiazole orange binds to the quadruplex-forming oligonucleotide with high affinity [23, 24]. In this investigation, TO was used for fluorescent intercalator displacement assay. Figure 3a and b indicate the effect of hemin on emission of the TO-G₄ complex in presence of KCl and NaCl. According to Fig. 3, the maximum wavelength of emission for TO without G₄, in the presence of KCl

Fig. 3 Fluorescence spectra of thiazole orange in presence of K^+ (a) and Na^+ (b) forms of the quadruplex DNA titrated with hemin; inserted figures indicate fluorescence intensity at various concentrations of hemin

and NaCl is 527 and 531 nm, respectively. After addition of G_4 DNA, the λ_{max} in the presence of KCl and NaCl became 532 and 534 nm, correspondingly, and the TO- G_4 emission intensity in KCl was greater than for NaCl. This dissimilarity is likely due to a difference in the binding of TO to two four-strand DNA formed in the presence of NaCl and KCl. Then, during increase of hemin to TO- G_4 , the maximum wavelength displacement in KCl changes to 530 nm and in NaCl to 528 nm, which corresponds to the emission wavelength of free thiazole orange. The intensity of TO emission at increasing concentrations of hemin is plotted in inserted Fig. 3a and b. The result indicates that in the presence of K^+ ion, when the ratio of hemin to TO is under 1, the intensity of TO emission increases. However, at higher concentrations of hemin, in which the ratio of hemin to TO is over 2, the intensity of TO emission decreases after addition of hemin to TO- G_4 . In the presence of Na^+ , the TO emission does not increase but, an increasing trend was observed after addition of hemin. The decrease in the emission spectrum of TO can be explained by displacement of hemin with TO. When the concentration of hemin is higher than TO, this fluorescent chemical compound is forced out from four-strand DNA bases and therefore its emission decreases.

In order to measure the Stern–Volmer constant, the fluorescence intensity of TO- G_4 in the presence of 150 μM of K^+ and Na^+ was obtained at maximum emission wavelength, before (F_0) and after (F) titration with the hemin complex solution. Stern–Volmer plots are shown in Fig. 4. The Stern–Volmer constant (K_{SV}) was measured using slopes of the linear plots. It was $2.5 \times 10^5 M^{-1}$ and $1.1 \times 10^5 M^{-1}$ in the presence of Na^+ and K^+ ions, respectively. This result shows that K_{SV} for the Na^+ form is larger than for the K^+ form indicating that the quenching of the TO- Na^+ form quadruplex is stronger than the TO- K^+ form after addition of hemin.

Fig. 4 Stern–Volmer plots for quenching of thiazole orange with hemin in presence of K^+ and Na^+ forms of the quadruplex DNA

3.3 Circular dichroism measurements

The structure of the G-rich DNA in the absence and presence of hemin was evaluated by circular dichroism spectroscopy (CD). Circular dichroism is a very sensitive technique for identifying alterations of structures of polynucleotides and is beneficial for a description of G-quadruplex-forming oligonucleotides [25–27]. The CD spectra of Na^+ and K^+ forms of the quadruplex DNA are shown in Fig. 5a. A positive peak at $\lambda = 264$ nm and a negative peak at $\lambda = 240$ nm are apparent, indicating a formation of parallel four-strand DNA in the buffer containing Na^+ and K^+ ions. As shown in Fig. 5a, the intensity of the G_4 spectrum at $\lambda = 260$ nm in the presence of Na^+ cation is more than the presence of K^+ cation. This situation is reversed at $\lambda = 240$ nm, where there are some differences in the structure of G_4 in both buffers containing NaCl and KCl in spite of similar folding. This may be the cause of structure rigidity. As shown in Fig. 5a and b, the CD spectra of both forms of four-stranded DNA do not change significantly in shape and peak locations due to interaction with hemin. Obviously, spectrum intensity decreases at $\lambda = 264$ nm in the presence of both Na^+ and K^+ ions but significant alteration in the intensity of the negative peak is observed only in the presence of NaCl. Accordingly, hemin interaction with four-strand DNA does not make substantial structural changes in the four-stranded DNA and only a slight change occurs in the conformation of both forms of G_4 -DNA upon interaction with hemin.

3.4 Catalytic activity

In this study, the change of luminol emission was measured in order to assess peroxidase activity of the DNAzyme. luminol substrate is oxidized in the presence of H_2O_2 , leading to production of light. The assay was performed in the presence and absence of four-strand DNA, in two buffers containing 150 mM KCl and 150 mM NaCl. The result (data not shown) indicated that the activity of hemin increases after binding to both forms of G_4 but, the

Fig. 5 Circular dichroism spectra of K^+ and Na^+ forms of the quadruplex DNA (a), K^+ form (b), and Na^+ form (c) in absence and presence of hemin

Fig. 6 VMD snapshots of the G-quadruplex DNA-hemin complex for (*left*) initial and (*right*) final conformation resulting from MD simulation

increased activity in presence of KCl ions is twice as much as NaCl ions, suggesting that the catalytic reaction in presence of KCl ions is faster than in the presence of NaCl ions.

3.5 Molecular modeling

The interaction between the quadruplex and hemin was visually evaluated (Fig. 6). The figure was prepared with VMD software after finishing the simulation. It shows that the distance between Fe^{3+} ion in the hemin molecule and oxygen in the phosphodiester bond of G_9 in DNA decreases, which indicates that these two molecules have an affinity to become closer to each other. The time-dependent distance between the mentioned atoms (data not shown) indicates that the distance between them, which was 20 Å in the beginning of the simulation, reduces to about 2 Å after passing about 320 ps and remains relatively constant in the remaining time of the simulation. It is reasonable to say that the

Fig. 7 RMSD plots of the quadruplex DNA before (*black*) and after (*gray*) binding to hemin

interaction can be attributed to electrostatic attraction; positive charges of Fe^{3+} are attracted by negative charges of oxygen in phosphate of sugar-phosphate backbone.

The root-mean-square deviation (RMSD) of the backbone atoms was also calculated and is presented in Fig. 7. The RMSD shows the mean spatial deviation of the atoms relative to their initial conformation. As shown, hemin leads to more change in the RMSD in the beginning of the simulation.

4 Conclusions

The interaction of hemin with K^+ and Na^+ forms of G-quadruplex DNA was investigated. Also, the peroxidase activity of hemin/G-quadruplex DNA was determined. The spectroscopic result indicated that the binding mode of hemin to G-quadruplex DNA is more likely outside binding. The result of molecular dynamic simulation demonstrated that hemin interacts with the end tetrad of the G-quadruplex DNA, which is consistent with the spectroscopic result. Circular dichroism spectra showed that hemin made no alteration in the type of DNA structure but, caused a change in the rigidity of the parallel G-quadruplex structure. This result is in close agreement with the binding mode of end-stacking, because it is expected that the integrity of the G-quadruplex structure is not destroyed upon stacking of hemin to the end tetrads of G-quadruplex DNA. The result of FID also has no contradiction with end-stacking, because TO interacts with the tetrad planes through end-stacking or intercalation. As noted, hemin competes with TO and drives it out.

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