Published in final edited form as:

J Mol Recognit. 2017 May ; 30(5): . doi:10.1002/jmr.2596.

Antichaperone activity and heme degradation effect of methyl *tert*-butyl ether (MTBE) on normal and diabetic hemoglobins

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Abstract

Because of the extensive use of methyl *tert*-butyl ether (MTBE) as an additive to increase the octane quality of gasoline, the environmental pollution by this compound has increased in recent decades. Environmental release of MTBE may lead to its entry to the blood stream through inhalation or drinking of contaminated water, and its interactions with biological molecules such as proteins. The present study was proposed to comparatively investigate the interactions of MTBE with hemoglobin (Hb) from diabetic and nondiabetic individuals using various spectroscopic methods including UV-visible, fluorescence, chemiluminescence, and circular dichroism. These results demonstrated the effects of MTBE on heme degradation of Hb and the reaction of these degradation products with water generating reactive oxygen species. Interaction of Hb with MTBE enhanced its aggregation rate and decreased lag time, indicating the antichaperone activity of MTBE upon interaction with Hb. Furthermore, the diabetic Hb showed more severe effects of MTBE, including heme degradation, reactive oxygen species production, unfolding, and antichaperone behavior than the nondiabetic Hb. The results from molecular docking suggested that the special interaction site of MTBE in the vicinity of Hb heme group is responsible for heme degradation.

Keywords

antichaperone; diabetes; heme degradation; hemoglobin; methyl *tert*-butyl ether (MTBE); ROS production

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1 INTRODUCTION

⁷ Human exposure to MTBE may result in headaches, vomiting, diarrhea, fever, cough, muscle aches, sleepiness, disorientation, dizziness, and skin and eye irritation.⁸ However, the detailed molecular and cellular mechanisms involved remain largely unknown.

Presence and accumulation of MTBE in the blood may result in its interactions with blood proteins. As hemoglobin (Hb) is one of the most abundant metalloproteins, and because of the active heme component of Hb,⁹ the interaction of this protein with MTBE needs investigation. Hemoglobin in the blood stream is responsible for transfer of oxygen from the lung to the body and carbon dioxide from the body to lung in the vascular system of vertebrates.¹⁰ Hemoglobin is a globular tetrameric protein with molecular mass of 64.5 kDa, composed of 2 pairs of polypeptide chains, 2 α chains with 141 amino acids, and 2 β chains with 146 amino acids.^{11,12} Four chains of Hb are held together through noncovalent interactions. Each of these chains contains a heme prosthetic group, a protoporphyrin ring containing a central ferrous atom (Fe^{2+}) for reversible binding to a molecule of O_2 or CO_2 .^{13,14}

In healthy people, 90%, 2%, 1%, and 7% of Hb belong to A1 ($\alpha_2\beta_2$), A2 ($\alpha_2\beta_2$), F ($\alpha_2\gamma_2$), and glycated forms of Hb, respectively. The binding of glucose to the β chain of Hb results in glycated hemoglobin A_{1c} (HbA_{1c}), which accounts for 5% of total Hb in nondiabetic people.¹⁵ Glycated HbA_{1c} level is primarily used for the determination of average plasma glucose concentration over a long period. Increasing levels of the glycated form of Hb in diabetes mellitus relates to the cardiovascular complications, and diabetic nephropathy and retinopathy.¹⁶ Advanced glycation end products (AGEs), resulting from the nonenzymatic glycation of proteins or oxidation of other biological macromolecules such as nucleic acids and lipids,¹⁷ are a heterogeneous, complex group of compounds that are formed mainly via the Maillard reaction.¹⁸ Prolonged exposure to AGEs plays an important role in the development and progression of the diabetes complications.¹⁹ However, little is known about the mechanisms involved and how these nonenzymatic glucose modifications impact protein function.

In molecular biology, molecular chaperones are proteins that assist protein folding by binding to the unfolded or misfolded chains and prevent their aggregation or reverse their misfolding.^{20,21} When the macromolecules have correctly completed the processes of folding and/or assembly by chaperones, they can perform their normal biological functions.²² However, in certain cases, some chaperones or other molecules, which are called antichaperones, facilitate the formation of protein aggregates.²³ In the presence of molecules with chaperone activity, increasing their concentration will decrease protein aggregation and increase the lag time. Under conditions where antichaperone activity dominates, increasing their concentration will increase aggregation and decrease lag time.²⁴ The aim of the present work was to comparatively study the mechanistic interaction of MTBE with Hb from normal and diabetic individuals and to elucidate the aforementioned interactions and their consequences by experimental and theoretical methods.

2 MATERIALS AND METHODS

2.1 Materials

Luminol (5-amino-2, 3-dihydro-1, 4-phthalazine), hydrogen peroxide (H_2O_2 , 30% solution standardized by a UV-Vis spectrometer at 240 nm), MTBE, and all phosphate buffer salts were purchased in analytical grade from Merck (Darmstadt, Germany). Gold(III) chloride hydrate (HAuCl_4) was purchased from Sigma (St Louis, Missouri). All solutions were prepared with double-distilled water.

2.2 Hemoglobin preparation

Human Hb was extracted from the blood of healthy and diabetic donors according to the previously reported methods.^{25,26} Blood was centrifuged to remove plasma components. The packed red cells were washed by adding 10 volumes of an isotonic saline solution (0.9% NaCl) and centrifuged at 4°C for 15 minutes at 10 000 rpm. After removing the supernatant, 5 volumes of phosphate buffer (200mM, pH 7.4) was added to the sample and centrifuged at 5000 rpm for 15 minutes. The washed packed cells were lysed with 5 volumes of deionized water and centrifuged at 4°C for 10 minutes at 18 000 rpm. In this step, stroma was discarded. The Hb solution was then brought to 20% saturation with ammonium sulfate, left standing for 15 minutes, and centrifuged at 2°C for 1 hour at 14 000 rpm. The recovered supernatant was then dialyzed by phosphate buffer (50mM, pH 7.4) at 4°C for 48 hours, which was changed every 7 hours.

2.3 Methods

2.3.1 Aggregation measurements—The aggregation of Hb (3.1 μM in 50mM phosphate buffer) in the presence or absence of MTBE were studied by monitoring the relative turbidity at a wavelength of 360 nm and temperature of 331 K using a UV-Vis Varian spectrophotometer (model Cary-100). Duration of the lag phase was obtained as the time intercept of fitted line to the linear portion of turbidity versus time plot, which appeared after the lag phase. The kinetic data were fitted to the model described by Wang and Kurganov.²⁷ In the case of first-order kinetic fitting of this model, effective first-order aggregation rate constant, k_1 , could be extracted from the slope of line of $\ln[(\tau_{\text{lim}} - \tau)/\tau_{\text{lim}}]$ versus $(t_0 - t)$ plot according to Equation 1:

$$\ln[(\tau_{\text{lim}} - \tau)/\tau_{\text{lim}}] = k_1(t_0 - t), \quad (1)$$

where τ is turbidity at time of t , τ_{lim} is the limiting value of turbidity as time tends to infinity, and t_0 is a lag time during which the absorbance is effectively zero.

2.3.2 Circular dichroism spectropolarimetry—For protein secondary structure determinations, far UV-CD spectra (195–260 nm) of samples were performed at 25°C using a JASCO J-810 spectropolarimeter. The spectra were recorded in a 1-mm quartz cell and step size of 1 nm and were deconvoluted using the CDNN CD Spectra Deconvolution Software (version 2.1). For this analysis, the concentration of Hb was 3.1 μM in phosphate buffer (50mM) by using a UV-Vis Varian spectrophotometer (model Cary-100).

2.3.3 Heme degradation analysis—Similar to the work of Nagababu and Rifkind,²⁸ in the current study, a fluorescence technique was used for detection of the heme degradation products of Hb during its interaction with MTBE. All fluorescence measurements were performed using a fluorescence spectrophotometer (Cary Eclipse, Varian Co). Fluorescent emission (Em) spectra were scanned from 330 to 600 nm at an excitation (Ex) wavelength of 321 nm and from 470 to 700 nm at an excitation wavelength of 460 nm, for Hb solutions with a concentration of 0.5 mg/mL, in the absence or presence of MTBE at 25°C in 50mM phosphate buffer at pH 7.4. In all fluorescence experiments, the width of both Ex and Em slits of the instrument was set at 10 nm.

2.3.4 Reactive oxygen species measurements—A chemiluminescence spectroscopy-based method was used for the determination of the reactive oxygen species (ROS) production capability of MTBE in its interaction with Hb.^{29,30} Experimental details of this test were as follow: 5 µL of HAuCl₄ solution (dissolved in distilled water) and 5 µL Luminol solution (dissolved in 100mM sodium carbonate buffer; pH 11) were injected into the buffer, Hb, or mixture of MTBE and Hb samples, and the chemiluminescence data were immediately recorded at a wavelength of 425 nm using a chemiluminescence spectrophotometer (Synergy H4 Hybrid Reader; BioTek, United States). The measurements were repeated at least 3 times.

2.3.5 Molecular docking—Molecular docking was performed using AutoDock 4.2 and AutoDockTools version 1.5.6 with standard parameters.³¹ Crystal structure of human Hb was obtained from protein data bank (pdb code 1GZX), ligand structure was constructed, and energy was minimized using PRODRG online server.³² All calculations were performed with the use of a PC with Intel Core i7 Processor. Images were generated with PMV.³¹

3 RESULTS

3.1 ROS measurements

The results of ROS production by incubation of MTBE with Hb from diabetic or normal individuals are summarized in Table 1. There was no ROS present in the buffer and mixture of buffer with MTBE. Thus, MTBE by itself does not produce ROS. Hemoglobin itself could produce a low amount of ROS in phosphate buffer solution. In the presence of MTBE, ROS production capability of Hb from both diabetic and normal blood strongly increased.³³ In addition, the ROS amount in solution increased with increasing concentration of MTBE. Hemoglobin from diabetic blood produced a greater amount of ROS than Hb from normal blood. This effect could be related to the glycation of Hb and glycoxidation effects of the protein.

3.2 Aggregation analysis

Thermal aggregation study of a protein, which is in fact a turbidity measurement at a wavelength of 360 nm, is a useful technique for measurement of the resistance against thermal denaturation. A thermal aggregation curve is composed of 3 phases: a lag phase, a logarithmic phase, and a plateau phase. A large lag phase and a slow slope logarithmic phase indicate a more stable protein.³⁴ With the increase of protein unfolding, thermal aggregation

of that protein occurs in shorter lag phase along with a fast slope.³⁵ Figure 1 shows the effect of MTBE on the aggregation of the Hb from diabetic and normal blood. Although there are some changes in the CD and fluorescence spectra of Hb during its interaction with MTBE, because there is no aggregation in the proteins, this effect could not be observed in the aggregation test during the lag time. As discussed above, the presence of MTBE in the vicinity of Hb could stimulate ROS production. As previously reported, such oxidative conditions could lead to protein unfolding, which subsequently results in the acceleration of the protein aggregation.³⁶

Figure 1 shows that in the presence of MTBE, the aggregation rate of Hb, from both diabetic and normal blood, increases. Aggregation rate of Hb from diabetic blood was greater than that of the Hb from normal blood. As discussed, the glycoxidation condition of Hb from diabetic blood results in overproduction of several sources of ROS, which in turn increases its aggregation rate compared with Hb from normal blood. However, at higher concentrations of MTBE, the aggregation rate differences between Hb's from diabetic and normal blood decreased, and the glycoxidation effects of AGEs vanished.^{17,37} At this level of MTBE, the production of ROS from the interaction of MTBE with Hb dominated and was the main contributing factor.

In addition to the increasing rate of the Hb aggregation, the presence of MTBE decreased the lag time of the aggregation in both the diabetic and normal Hb's. Decrease in lag time for protein aggregation indicates that the protein is undergoing denaturation in its structure.³⁵ Thus, the presence of MTBE caused the unfolding of Hb.

3.3 CD analysis

The CD was used to obtain a precise survey of the secondary structure changes of the Hb, from normal and diabetic samples, in the presence of MTBE. The results showed that the presence of MTBE caused secondary structural changes in Hb's. However, the diabetic Hb underwent more structural changes than normal Hb (Figure 2). In both of these samples, the presence of MTBE decreased the alpha helix contents of the Hb's and increased the random coil contents (ie, protein unfolding). In addition, the random coil contents of the protein increased with the increase in concentration of MTBE (see Table 2).

From the results of the ROS, aggregation, and CD spectroscopy analysis, one could conclude that the presence of MTBE increased ROS content of the protein solutions, and the ROS in turn accelerates protein aggregation by increasing the random coil contents of the proteins. As previously indicated, MTBE or Hb alone generates limited or no ROS, but the interaction of Hb-MTBE enhanced ROS production. These were mainly attributed to the Hb aggregation and heme degradation mediated by interaction with MTBE. This is reported in the literature as the protein aggregation induced ROS production.³⁸

3.4 Heme degradation

Figure 3 shows heme degradation at different excitation wavelengths of 320 and 460 nm, for Hb's from both normal and diabetic samples. There are some heme degradation products from the normal and diabetic Hb's, which were intensified upon interaction with MTBE. Sigmoidal trends of the aforementioned interactions, at both excitation wavelengths of 320

and 460 nm, are shown for normal and diabetic Hb's (Figure 3). Thus, the diabetic Hb was more susceptible to heme degradation in the presence of MTBE than normal Hb.

3.5 Molecular docking

To better understand the mechanisms of Hb interactions with MTBE, we studied the aforementioned interactions between MTBE and Hb through molecular docking. The molecular docking calculations showed that MTBE mainly interacted with the amino acids adjacent to the heme group of Hb (Val680, Val677, and Ala681) (Figures 4 and 5). After 100 runs, we observed that MTBE could interact through hydrophobic interactions with amino acids in the vicinity of the heme group.

4 DISCUSSION

Methyl *tert*-butyl ether belongs to a large family of oxygenated ethers including ethyl tertiary butyl and methyl tertiary amyl ether that came into use as motor vehicle fuel additives in the late 1970s. The principal sources of air pollution are motor vehicle emissions, and production, blending, distribution, and storage of gasoline. However, the main sources of water contamination are due to MTBE deposited from air, gasoline spillage during different phases of production, and leakage from storage tanks. Occupational MTBE exposure therefore affects all workers exposed to gasoline and also the general population exposed to MTBE present in the air during automobile refueling. Chronic MTBE exposure in rodents has been related to a statistically significant increase in renal tubular cell and testicular Leydig cell tumors in males and leukemia and lymphomas in females. Research on humans has failed to provide conclusive results, although there have been reports of nephrotoxicity, depression of the central nervous system, migraine, tremors, ataxia, cough, and dyspnea in exposed participants. To identify the molecular interaction of MTBE with human proteins, we previously reported that MTBE promotes the molten globule-like state and structural changes in insulin.³⁰ These findings confirmed the potential toxicity of MTBE and might be relevant to understanding the effects observed *in vivo*.^{5,39}

So as to clarify the sensitivity of a diabetic person to MTBE, in the present work, interaction of MTBE with Hb from normal and diabetic individuals were comparatively investigated using different methods. The results of molecular modeling and fluorescence studies indicated that MTBE cause heme degradation upon interaction with Hb. Molecular docking studies showed that MTBE is located in a small cavity adjacent to the heme group, which is composed of nonpolar amino acids (Val680, Val677, and Ala681) (Figures 4 and 5). Hydrophobic interactions of MTBE with indicated amino acids could be an important factor, which change the heme binding packed conformation. This conformational change may be the main reason for heme degradation. The fluorescence spectroscopy analysis, after treating Hb with different concentrations of the MTBE, showed heme degradation products formed in the solution. Increasing the concentration of MTBE produced more heme degradation products by removing more heme groups from the protein structure.

The extruding heme group probably made Hb structure mutable and triggers the denaturation and unfolding process. In addition to structural changes due to removing heme group, the heme degradation products are capable of producing ROS, which could be

responsible for further denaturation and unfolding of Hb molecules.^{28,40,41} The relationship between ROS and protein denaturation can be explored by CD techniques. Our results showed an alpha helix to random coil transition in both normal and diabetic Hb's. The alpha helix is often sustained by hydrogen bonds, which was created between hydroxyl and amine groups. The ROS-generating compounds that formed after heme degradation could alter the hydroxyl groups participating in an alpha helix structure and change them to a carbonyl group.^{42,43} This chemically altered functional group can no longer form hydrogen bonds and, therefore, may cause an alpha helix conformational change and random coil transition. As a result of this structural transition, protein denaturation and unfolding were most intensified in diabetic Hb than in normal Hb. Moreover, this can be seen from aggregation test that diabetic Hb aggregated more rapidly than normal Hb.

According to data reported by Mullarkey and co-workers,⁴⁴ non-enzymatic glycation of some reactive amino groups in proteins can increase free radical production at physiological pH. Thus, glycated Hb may produce more free radicals than nonglycated Hb in the presence of MTBE by triggering ROS production. This will intensify protein structural changes and aggregation in Hb from diabetic samples compared with those in normal Hb.⁴⁵ Scheme 1 indicates the mechanism of Hb interaction with MTBE.

5 CONCLUSION

In this work, mechanism of the destructive effects of MTBE on Hb was elucidated. We showed that MTBE induced heme degradation through its interaction with specific sites in Hb. The heme degradation products, in turn, reacted with water and produced ROS. The presence of ROS in protein solutions induced protein unfolding and aggregation (antichaperone activity). Unfolding protein itself increases ROS contents of the protein solution, and in this way, the protein unfolding and aggregation accelerate. Because of the glycoxidation effects, unfolding and aggregation of diabetic Hb were severe in comparison with those of normal Hb. In summary, the results of this work indicated the destructive effects of MTBE on Hb with the emphasis that a diabetic condition would be at a higher risk in the presence of MTBE.

Acknowledgments

The support of the following are gratefully acknowledged: University of Tehran; Office of Health, Safety and Environment (HSE) Oil Ministry, Bandar Mahshahr, Iran; Center of Excellence in Biothermodynamics (CEBiotherm); Iran National Science Foundation (INSF); Iran's National Elites Foundation (INEF); Center for International Scientific Studies and Collaboration (CISSC)-Ministry of Science, Research and Technology in Iran; UNESCO Chair on Interdisciplinary Research in Diabetes at the University of Tehran; and Iran Society of Biophysical Society.

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FIGURE 1.

Thermal aggregation of the diabetic and normal hemoglobins in the absence and presence of methyl *tert*-butyl ether (MTBE) with different concentrations

FIGURE 2.

Circular dichroism spectra of the diabetic and normal hemoglobins in the absence and presence of methyl *tert*-butyl ether (MTBE) with different concentration

FIGURE 3.

A and B, Heme degradation fluorescence test results for normal and diabetic hemoglobins at $\lambda_{\text{ex}} = 320$. C, Comparison of heme degradation trends of normal and diabetic hemoglobins at $\lambda_{\text{ex}} = 320$. D and E, Heme degradation fluorescence test results for normal and diabetic hemoglobins at $\lambda_{\text{ex}} = 460$. F, Comparison of heme degradation trends of normal and diabetic hemoglobins at $\lambda_{\text{ex}} = 460$ (direction of arrows show the increasing direction of the methyl *tert*-butyl ether [MTBE] concentration)

FIGURE 4.

Three-dimensional presentation of heme group position of the hemoglobins and methyl *tert*-butyl ether (MTBE). MTBE, heme group, and oxygen molecule are presented as purple VDW view, yellow line, and red VDW view, respectively. As it can be seen, hydrophobic tail of MTBE was positioned near the heme group

FIGURE 5.

Line representation of interaction site of methyl *tert*-butyl ether (MTBE) and hemoglobins. As it can be seen from this figure, MTBE was positioned in a hydrophobic cavity composed of nonpolar amino acids. This cavity is adjacent to the heme group, and MTBE binding could alter the sensitive position of the heme group and finally may cause the release of the heme group from protein structure to the surrounding solution

SCHEME 1.

Schematic illustration of the interaction mechanism of methyl *tert*-butyl ether (MTBE) with hemoglobins (the protein chains of hemoglobin [Hb] are presented as colored ribbons (with yellow, blue, red, and green colors), heme parts of Hb are presented in black lines, and Fe²⁺ ions are presented as ochre spheres)

TABLE 1

ROS measurements through chemiluminescence spectroscopy

Sample	ROS Amount, μM	Sample	ROS Amount, μM
Buffer	0	Buffer + 500 μM MTBE	0
Normal Hb	0.011	Diabetic Hb	0.239
Normal Hb + 100 μM MTBE	1.002	Diabetic Hb + 100 μM MTBE	1.759
Normal Hb + 200 μM MTBE	3.368	Diabetic Hb + 200 μM MTBE	4.268
Normal Hb + 300 μM MTBE	4.499	Diabetic Hb + 300 μM MTBE	5.667
Normal Hb + 400 μM MTBE	5.387	Diabetic Hb + 400 μM MTBE	7.309
Normal Hb + 500 μM MTBE	6.263	Diabetic Hb + 500 μM MTBE	8.464

Abbreviations: Hb, hemoglobin; MTBE, methyl *tert*-butyl ether; ROS, reactive oxygen species. All samples were prepared at 25°C in 50mM phosphate buffer at pH 7.4.

TABLE 2

Secondary structure contents of normal and diabetic Hb's in the absence and the presence of MTBE

	Normal Hb, %	Normal Hb + MTBE (300μM), %	Diabetic Hb, %	Diabetic Hb + MTBE (300μM), %
Alpha helix	61.08	52.27	54.72	46.24
Antiparallel	3.04	4.07	4.57	4.96
Parallel	4.12	4.71	4.97	5.83
Beta turn	11.76	13.73	13.91	14.64
Random coil	20.29	25.21	21.93	28.34

Abbreviations: Hb, hemoglobin; MTBE, methyl *tert*-butyl ether; ROS, reactive oxygen species.