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Reactivity of glass-embedded met hemoglobin derivatives towards external NO: implications for nitrite-mediated production of bioactive NO

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Abstract

Many protein reactions are exceedingly difficult to dissect under standard conditions due to low concentrations of reactants and intermediates. A case in point, are several proposed reactions of hemoglobin with both nitrite and nitric oxide. In the present work, glassy matrices are used to dynamically control the rate at which externally introduced gaseous NO accesses and reacts with several different met Hb derivatives including the nitrite, nitrate and aquomet forms. This novel yet general approach reveals a clear difference between nitrite and other ligands including nitrate, water and an internal imidazole. Whereas for nitrate, water and the internal distal imidazole, the observed spectral changes indicate that NO entering the distal heme pocket is effective in displacing these ligands from the ferric heme iron. In contrast, when the ligand is nitrite, the resulting initial spectra indicate the formation of an intermediate that has distinctly ferrous-like properties. The spectrum and the response of DAF fluorescence to the presence of the intermediate is consistent with a recently proposed nitrite anhydrase reaction. This proposed intermediate is especially significant in that it represents a pathway for a nitrite-dependent catalytic process whereby Hb generates relatively long lived bioactive forms of NO such as S-nitrosoglutathione. The failure to form this intermediate either at low pH or when the glass is extensively dried is consistent with the requirement for a specific conformation of reactants and residue side chains within the distal heme pocket.

Introduction

Many physiologically important protein-based reactions are exceedingly difficult to dissect in that contributing species and intermediates are often present at levels that make biophysical characterization problematic. A case in point are several of the proposed reactions of hemoproteins such as hemoglobin and myoglobin with nitrite and nitric oxide (NO) that have been invoked to account for the production of bioactive forms of NO capable of vasodilation^{1–8}. An understanding and clear exposure of these reactions is an essential element to resolve the ongoing debate as to whether and through what mechanisms Hb can function as a non-NOS (nitric oxide synthase) source of bioactive NO and thus confer upon red blood cells^{3,4,6,8} and acellular Hb based oxygen carriers (HBOCs)^{9,10} the capacity to act as vasodilators.

The presented work shows how glassy matrices can be used to systematically compare reactivity of gaseous NO with different met Hb derivatives including the nitrite, nitrate and aquomet derivatives. This approach allows one to probe aspects of these reactions normally not amenable to detailed biophysical study in solution. The ultimate objective is to focus on the NO-mediated reduction of ferric derivatives of Hb in the presence of nitrite anions. This

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reaction is significant in that it has been proposed² as a major element in the pathway for generating either NO+ or N₂O₃, two potent nitrosating agents of thiol-containing peptides such as glutathione as well as the intrinsic reactive thiol (Cys β93) on Hb⁷. The work specifically seeks to identify intermediates that function both as relatively stable forms of heme-associated NO and as a source of either NO+ or N₂O₃. The standard NO derivatives of Hb are not viable contenders for this function. The NO derivative of met Hb is unstable due to a high rate of spontaneous dissociation and the NO derivative of ferrous Hb is too stable with too low a rate of NO dissociation. Recent studies⁸ have purported to have identified a possible role for the Fe(+2)-NO+ resonance structure associated with the ferric NO derivative (Fe(+3)-NO) in a possible mechanism for producing a long lived heme-associated form of bioactive NO. The present work addresses the formation of such intermediates as a function of different ligands bound to the ferric heme iron.

This work builds on earlier findings that glassy films derived from trehalose and trehalose/sucrose can dramatically slow down protein conformational dynamics^{11–16} including those that are likely to mediate NO reactivity with heme bound ligands. In this study, this property is used to control access to the heme with respect to NO diffusing within the glass.

The above properties are utilized in developing a strategy for protocols that allow for controlled access of NO into the distal heme pocket of Hb under conditions where there is nitrite, nitrate, water or imidazole (from His E7) bound to the ferric heme iron. Our approach, shown in Fig. 1, is to first prepare a thin glassy layer lining the inside lower third of an optical quality NMR tube that contains a substantial population of met Hb with nitrite, nitrate or water as the sixth ligand. High non-physiological concentrations of ligand are used to insure that the populations being probed are homogeneous. The issue being addressed is the reactivity of a specific species which may occur only as a minority species under physiological conditions. Unless otherwise stated the initial solutions are all at pH 7.5. These glassy samples are flushed with and stored under either dry argon or nitrogen. The experiment begins when the samples are flushed with dry gaseous NO that had been initially passed through a saturated KOH solution to remove higher oxides of nitrogen. The visible absorption spectrum primarily in the Q band regions is focused upon in that it is highly sensitive both to the nature of ligand coordinated to the heme and to the redox state of heme.

Additionally fluorescence from diaminofluorescein (DAF) is used to detect the formation of nitrous anhydride (N₂O₃). DAF is typically used as a fluorescent indicator with good specificity, sensitivity, and simplicity of use for measuring NO¹⁷. It was developed from the observation that aromatic amines react with NO in the presence of dioxygen to produce triazenes¹⁸. Similarly, triazole ring compounds are generated when aromatic vicinal diamines react with NO under neutral conditions in the presence of dioxygen. The proposed reaction mechanism involves the oxygen mediated formation of nitrous anhydride (N₂O₃), a potent nitrosating agent¹⁹. The DAF-2 fluorophore which is used in the present study is itself only a weakly fluorescent reagent that contains an aromatic vicinal diamine that undergoes a conversion to its fluorescent triazole derivative, DAF-2T, in the presence of N₂O₃. The nitrous anhydride is able to react with the DAF-2 in a N-nitrosation reaction to generate a triazole ring (a triazolofluorescein), thereby significantly enhancing the fluorescent signal by a factor of 100. This signal can be easily observed with an excitation wavelength of 495 nm, giving an emission maximum at 515 nm. While the absorption maxima does not significantly change in the DAF-2 reaction with the nitrous anhydride, the quantum efficiency of the dye is increased, leading to significant amplification of the emission signal coincident with the level of N₂O₃ in the sample. In the absence of dioxygen the DAF-2 is not responsive to NO. It has been shown that it does not respond to other commonly encountered oxidized NO species and reactive oxygen containing species (e.g. NO₂⁻, NO₃⁻, O₂⁻, H₂O₂, and ONOO⁻)¹⁷. Thus under the

conditions of our experiment, the increase in DAF-2 fluorescence is overwhelmingly attributable to the formation of nitrous anhydride (N_2O_3).

Experimental procedures

Materials

Hemoglobin, trehalose, sucrose, sodium nitrite, sodium nitrate, 4,5-Diaminofluorescein (DAF-2) were all obtained from Sigma-Aldrich (Missouri, USA), polyvinyl alcohol (PVA) was obtained from DU PONT, Wilmington DE, 99% pure nitrogen, argon were obtained from (Tech Air, USA) and gaseous nitric oxide was obtained from (PaxAir, USA). Hemoglobin prepared and purified directly from whole blood was generously provided by Dr. Seetharama Acharya. The met derivative of Hb was prepared using potassium ferricyanide followed by dialysis.

Methods

Stock solution of 80:20 mg/ml of trehalose:sucrose in deionized water were used to prepare glassy films. Sodium nitrite and sodium nitrate were added to the stock solutions to create solutions with final concentrations of nitrite and nitrate of 100 mM. The prepared stock solutions of Hb powder in 50 mM tris(HCl) is centrifuged to get a clear solution and the pH adjusted to either 7.5 or 6.5 according to the experimental requirements. Aliquots of stock aquomet Hb solutions (either from the Hb powder or the whole blood-derived protein) in 50 mM tris(HCl) (either pH 7.5 or 6.5) are added to the above solutions to achieve a final protein concentration of 0.25 mM. The pH of the final stock solutions were at either 7.5 or 6.5.

Glass preparation

Small aliquots of the resulting solutions were poured into a 1 cm diameter quartz NMR tube and mounted on spinning setup (Princeton Photonics Inc. Model: Raman 101). The NMR tube is spun a frequency that results in the solution forming a thin layer on the lower one fourth of the inner wall of the NMR tube. As the tube is spinning the solution is being dried with continuous purge of either nitrogen or argon gas. After the sample has formed a dry glassy film, the tube is sealed with a rubber septum and filled with nitrogen. For making extremely dry samples, the above protocol is modified in that the purging gas is first passed through a drying column containing desiccant to reduce the moisture content of the gas. The samples were stored in a dessicator for aging for a week or longer.

The reactions of the glass embedded met Hbs with NO are initiated by purging the glass containing tubes with gaseous nitric oxide that was first bubbled through a saturated KOH solution and then through a drying column filled with desiccant. After the sample is flushed with NO, a series of visible absorption spectra are generated on a PERKIN-ELMER (Lambda-2) UV-Vis absorption spectrometer as a function of time. In several instances, the protein containing layers were dissolved and the UV/Vis spectrum generated.

DAF Fluorescence

Dry thin glassy films derived from a trehalose:sucrose:PVA mixture (80:20:1 mg/ml) containing met Hb and sodium nitrite were prepared in quartz NMR tube as described above. The PVA was included in the formulation to provide enhanced structural integrity with respect to the rate at which the glass dissolves upon exposure to aqueous buffer. The samples were flushed with nitrogen and then filled with nitric oxide that had been passed first through the KOH solution and then a drying column. The NO-loaded samples were allowed to evolve for several hours until the desired intermediate spectrum (band positions at 538nm and 568nm) or final spectrum (542 nm and 572 nm) was observed. At these points as well as for the control

that had not been exposed to NO, the samples were purged thoroughly with argon gas for 20 minutes to get rid of the unbound nitric oxide and also to ensure an anaerobic environment. The absorption spectra were again generated to establish that the desired population remained stable during the purging process. One ml of stock solution of 10 μ M DAF-2 in tris ((HCl) 50mM, pH 7.5) that was purged thoroughly with argon gas was then added to the above glassy samples under an anaerobic nitrogen atmosphere. After allowing the DAF containing solution to remain in contact with the glass for one minute, the rigorously sealed NMR tube was inverted thus allowing a clean separation between the solution and the still intact glass. At this point the fluorescence from the DAF containing solution is acquired on a PTI Quanta Master Model QM-4/2000SE enhanced performance scanning spectrofluorimeter. The fluorescence emission, excited using a 495 nm excitation, was collected and scanned at 90° to the excitation. Once the tube was turned over and set into the fluorimeter, it was not disturbed until after the temporal sequence of scans was complete. In addition to the tube samples that included the initial met nitrite derivative, the “intermediate” and the ferrous NO derivative, the time dependent changes of the fluorescence were also generated for similar samples of DAF-2 exposed to an atmosphere of NO under anaerobic conditions and for a DAF-2 solution exposed to a glassy sample containing just 100 mM sodium nitrite. The tubes proved to be fully capable of maintaining the anaerobic conditions as reflected both in the stability of low intensity signal in the presence of NO under the sealed conditions and the large increase in fluorescence intensity when a air is allowed into the tube through a pin prick.

Results

A comparison of the response of the nitrite, nitrate and aquo derivatives of glass embedded met Hb to added gaseous NO

The top panel of Fig. 2 shows the Q band heme absorption spectrum of glassy samples of three different derivatives of met Hb. One sample was prepared without the addition of potential met Hb ligands other than water. The other two samples were prepared in the presence of either 0.1 M nitrite or 0.1 M nitrate. Prior to formation of the glass, the sample without additions and the sample with nitrate both manifested a spectrum consistent with a population that was overwhelming that of the aquomet Hb derivative indicating that water is the sixth ligand of the ferric heme iron under those conditions. The spectrum of the nitrite containing sample was consistent with a large population of the nitrite met derivative of Hb. Upon forming a glassy film, the three samples yielded the spectra shown in the top panel of Fig. 2. The spectrum of the sample without additives is still consistent with that of the aquomet derivative. The nitrite and the nitrate containing samples now both reflect large populations of met Hb with nitrite and nitrate as the sixth ligand, respectively. As the nitrate-containing sample dried, there was a progressive loss of the aquomet spectrum which was ultimately replaced by the spectrum shown in the top panel which matches a previously reported spectrum of the nitrate derivate generated in solution using a much larger excess of nitrate²⁰. The formation of the nitrate derivative in the glass can be readily attributed to water becoming less of a competitive ligand due to the osmotic stress from the glass^{21,22}. The bottom three panels show the succession of changes occurring within the first hour after adding the gaseous NO. Initially, both the aquomet and the nitrate met glassy samples show the appearance of a Q band spectrum bands characteristic of the met NO Hb derivative. The spectrum shows the characteristic deep valley between the two peaks at 535 and 565 nm. In contrast the met nitrite samples displays an initial spectrum both with bands that are clearly red shifted relative to those of the met NO derivative and devoid of the deep valley between the two Q band peaks that occurs in the NO met Hb spectrum. Over the one hour, both the nitrite and aquo met samples both show spectra that are consistent with the formation of the ferrous NO derivative of Hb; whereas, on this same time scale, the spectrum of the nitrate sample does not evolve beyond that of the met NO derivative. Both the aquo and nitrite met samples end up as ferrous NO species. The aquomet sample is

initially converted to the met NO derivative which subsequently undergoes reduction to the ferrous NO derivative. In contrast, the nitrite met derivative goes directly to an intermediate (*vide infra*) that is distinctly different from the met NO derivative which then evolves into the standard ferrous NO species for either T or R state forms of Hb (see Figure 1s and 2s in the Supplemental Data section).

Figure 3 compares the NO-induced spectral evolution of aquo and nitrite met Hb samples in glassy matrices that have been allowed to dry for a slightly longer time than for the samples associated with Fig. 2. In this case, the NO-induced evolution is more protracted, extending over a 92 hour time window. As in Fig. 2, the aquo met sample when exposed to NO, evolves first into a met NO population that subsequently undergoes reduction as is evidenced by the resulting ferrous NO spectrum. The addition of NO to the nitrite sample results in a decrease in the ~ 620 nm met Hb band and the formation of a Q band spectrum that resembles the ferrous NO spectrum but is blue shifted by several nanometers as can be seen in the figure (~538/568 nm *vis a vis* ~543/573 nm). This intermediate spectrum smoothly evolves into the ferrous NO spectrum with time.

The effect of pH on the NO-mediated reduction of the nitrite met Hb derivative in a glassy matrix

The pH dependent response of the spectrum of the nitrite met derivative of Hb in a glassy matrix to the addition of NO is shown in Fig. 4. Whereas for the pH 7.5 sample (panel b), the spectrum shows evolution towards the ferrous NO derivative over the 320 minute observation window, the pH 6.5 sample remains stable as the met NO derivative (panel a). With time the spectrum of the pH 6.5 sample begins to show evidence of heme degradation as reflected in the appearance of a broad back ground and a peak at ~ 600 nm which can be seen starting to develop in the later traces in Fig 4a.

The effect of enhanced drying of the glass

Fig. 5 compares the NO-induced spectral evolution of three met Hb samples. In the top panel (a), the sample is a nitrite met Hb sample in a moderately dry glass; whereas, the middle and bottom panels show results from very dry glassy samples of nitrite met Hb and nitrate met Hb, respectively. It can be seen that the clearly evident evolution towards ferrous NO Hb occurring with the nitrite sample in the moderately dry glass does not occur for the very dry nitrite sample. The very dry nitrate sample does show the progressive appearance of the met NO Hb spectrum indicating that NO can still access the distal heme pocket of met Hb in the very dry glass.

If the added gaseous NO can access the distal heme pocket even for the very dry glassy samples, the question arises as to why the nitrate sample shows clear indications of NO binding whereas the nitrite sample does not. Nitrate is a very weak ligand and as a consequence it is plausible that when NO enters into the distal heme pocket of the nitrate met Hb molecule, the NO easily displaces the nitrate ligand. In contrast nitrite is a stronger ligand and as a result may take longer to displace due to a slower spontaneous dissociation for nitrite *vis a vis* nitrate. The NO-initiated time evolution of the absorption spectrum from very dry glassy samples of a nitrite met Hb and of an aquo met Hb that has become a hemichrome due to the excessive drying are shown in Figure 6 and Figure 7 respectively. The hemichrome heme has as sixth ligand, the imidazole from the distal histidine. It has been shown that under high osmotic stress, the water that is sixth ligand associated with the aquo met derivative is replaced by the distal imidazole^{21,22}. In both cases the spectra show that NO eventually does bind to the heme. In the case of the very dry glassy nitrite derivative, the resulting spectrum is that of the NO met derivative. The hemichrome sample also shows a clear spectrum characteristic of the NO met derivative (535 and 565 nm) but there is continued slow evolution (red shifting of the bands) on the shown

time scale consistent with evolution towards either an intermediate or the ferrous NO derivative.

DAF-2 fluorescence increases when the intermediate is formed

No or negligible increase in the fluorescence from DAF-2 is observed when the DAF solution is exposed to NO, nitrite, met nitrite in the glass, and ferrous NO in the glass. In marked contrast the DAF-2 fluorescence from samples (5 in total) that progressed to the “intermediate” show a steady increase in intensity over a several minute time scale as shown in Fig. 8.

Discussion

Protein reactivity in a glassy matrix

In earlier work^{21,22}, it was shown that hemeproteins can undergo redox reactions in glassy matrices that are initiated through thermal or photo generation of electrons. In the present study, the results show that NO introduced as an external gas can diffuse into the glass, access internal sites within Hb and initiate internal redox reactions at the heme site even at ambient temperatures that are well below the glass transition. These results are indicative of several categories of conformational dynamics remaining active even below the glass transition of the trehalose/sucrose matrix.

Two categories of protein dynamics remain active for Hb in the trehalose/sucrose glass at ambient temperature

As noted in the introduction, it has been shown by several groups that protein dynamics associated with either Mb or Hb are significantly damped in glassy matrices. It has been shown that there are different categories of protein dynamics that can be organized hierarchically based on which class of solvent dynamics they are slaved to^{23–27}. We have extended this concept to define three protein dynamic states based on which category of dynamics is active during a given measurement^{28–30}. The B, C and D protein dynamic states refer to protein dynamics that are respectively: i) not slaved to the solvent; ii) slaved to small amplitude solvent dynamics referred to as the β fluctuations (relaxations); and iii) slaved to large volume changing fluctuations known as α fluctuations (or relaxations). In the glass, the C state dynamics which facilitate ligand or substrate diffusion within the protein are still active on the nano to microsecond time scale^{16,28–30} unless the glass is extremely dry¹⁵. The large volume changing fluctuations associated with the D state are needed to allow access into and out of the distal heme pocket of most Hbs and Mbs. At ambient temperatures D state dynamics for Hb and Mb in a glassy matrix are significantly damped such that they are in effect not active during conventional photodissociation/recombination studies^{11,13–16,28–31}. With increasing temperatures, especially as the glass transition temperature is approached, the influence of the D state dynamics becomes apparent at progressively shorter time periods^{29,30}.

In the present study, the observation that NO can enter the distal heme pocket, displace water or nitrate as the heme sixth ligand indicates that on the time scale of our measurements both C and D state dynamics are active within the glassy matrices used in this study. The time scale that defines the protein dynamic state in the present study is very much longer than those associated with the ligand recombination experiments discussed in the previous paragraph.

NO reactivity with met Hb: the role of the sixth ligand

The results for the least dry glasses prepared from solutions at pH 7.4, indicate that the fate of the NO entering the distal heme pocket of met Hb is a function of the sixth ligand. When the sixth ligand is water, nitrate or the E7 imidazole side chain, the entering NO displaces the sixth ligand and binds to the ferric heme iron yielding the standard met NO Hb spectrum. Under

these same conditions, NO does not comparably displace the heme bound nitrite as is evidenced by the absence of a discernable met NO spectrum when the influence of NO is first detected. Instead, the NO encountering the heme bound nitrite generates an initial spectrum that is intermediate in appearance between the met NO and ferrous NO spectra. This intermediate spectrum could not be simulated using a simple linear combination of the met NO and ferrous NO spectra. Figure S1 and S2 in the supplemental information section show how the spectrum of the “intermediate” is clearly distinct from ferrous NOHb samples generated in solution (pH 7.5 and pH 6 + IHP) and in the glass via multiple pathways. A detailed treatment of the spectrum of the “intermediate” will be presented in a subsequent manuscript describing the formation of the intermediate in both solution and sol-gels.

Low pH inhibits formation of the met nitrite plus NO intermediate

In contrast to what occurs in the glass at pH 7.5, the entry of NO into the distal heme pocket for a met nitrite Hb sample in a glass prepared at pH 6.5 does result in a seemingly straight forward displacement of the nitrite by the NO resulting in the formation of the standard met NO Hb derivative. A proposed nitrite anhydrase reaction² can account for this pH dependence. In this proposed reaction, the heme-nitrite complex exists in a dynamic equilibrium between a ferric nitrite form which has a negative charge localized on the ligand and ferrous nitrite radical where the charge is localized within the heme (resulting in ferrous heme) and the bound nitrite now exists as a radical. The NO which is a radical combines with the ferrous heme bound nitrite radical to produce N_2O_3 bound to a ferrous heme iron. It is plausible that at the lower pH, the protonated form of the distal histidine stabilized the ferric-nitrite species which decreases the probability for the reaction of the NO radical with the ferrous nitrite radical. As a result the NO displacement of nitrite from the ferric heme becomes the dominant process.

Conformational dynamics are required to form the nitrite anhydrase intermediate

The results obtained for the very dry glasses indicate that NO can still access the distal heme pocket of the different met Hb derivatives. It is clear that NO displaces the nitrate and E7 imidazole ligands on a faster time scale than nitrite. Under these conditions for all of the samples, the initial spectra reflecting NO activity are indicative of a simple displacement of the ligand with the formation of the met NO derivative. It was noted above that the slower displacement of the nitrite could be a result of a greater degree of stabilization of the nitrite leading to a slow dissociation rate. The additional question is why under the dry glass conditions, does the nitrite get displaced by NO whereas the less dry glass samples show clear evidence of an intermediate being directly formed. A plausible explanation is likely related to observations that as a glass loses water, the amplitude of large amplitude conformational dynamics of embedded proteins are increasingly damped. If the formation of the intermediate requires the accessing of a specific conformation of substrates and residue side chains within the distal heme, it is then probable that sufficient damping of conformational dynamics decreases the probability of accessing this required reactive configuration of substrates (NO and nitrite) and residue side chains. We propose that under conditions where the damped dynamics sufficiently slow the formation of the intermediate, the direct displacement reaction can become the dominant pathway. When under dry conditions, the E7 imidazole is the sixth ligand, the NO induced progression shows the initial formation of the met NO species which is consistent with a direct displacement. In contrast to the situation with met nitrite in a very dry glass, where the met NO derivative appears to be a relatively stable end point (Fig 6, Fig 7 day spectrum), here the met NO Hb spectrum evolves into what appears to be an intermediate spectrum (see Fig 7, the 4 day spectrum) suggestive of what was seen for the nitrite intermediate observed for the less dry samples. A plausible explanation is that for the hemichrome species, once the NO displaces the distal E7 imidazole resulting in the formation of a met NO derivative, there is a slow but small adjustment of the distal E7 imidazole that alters the proposed^{4,8} resonant $\text{Fe}(+3)\text{-NO} \leftrightarrow \text{Fe}(+2)\text{-NO}^+$ species so as to increase the amplitude of the ferrous-like

component which may also function as a relatively long lived source of bioactive NO. Conversely the stability of the ferric NO product for the NO exposed met nitrate species (See Fig. 2) may be the result of the dissociated positively charge nitrate remaining in the distal heme pocket and favoring a resonant form that is overwhelmingly ferric NO vis a vis the electrostatically less favorable ferrous-NO⁺.

Evidence that the intermediate is associated with N₂O₃

The DAF-2 results show that the formation of the intermediate derived from the addition of NO to the met nitrite derivative is likely associated with the formation of N₂O₃. The DAF-2 effect occurs for samples that have most if not all of the unbound gaseous reactants and products removed. This observation and the ferrous-like spectrum of the intermediate are consistent with the proposed mechanism² that has NO reacting with met nitrite Hb to form a ferrous species with a heme-bound N₂O₃. At this point it is not clear as to whether electrons from the Cys β93 sulfhydryl side chains are contributors to this process. Preliminary attempts to test the role of these sulfhydryls by using NEM modified Hb proved difficult due to the effect of the glass on the this protein; however, the basic reaction is also seen in sol-gel matrices for both NEM modified and wild type HbA (unpublished results).

Conclusions

Contrary to the reasonable expectation that protein reactivity towards an external substrate would be essentially shut down in a glassy matrix, the present result shows that external gaseous NO not only diffuses through a glassy matrix but also accesses internal sites within a glass embedded protein albeit on much slower time scales than in solution. The slow down in protein dynamics within the glass allows for the following of protein reactions and processes that are difficult to study under solution phase conditions. When applied to reactivity of different met Hb derivatives to external NO, this approach reveals a clear difference between nitrite and other ligands including nitrate, water and an internal imidazole. Whereas for nitrate, water and the internal imidazole, the observed spectral changes indicate that when NO enters the distal heme pocket, it is effective in displacing these ligands from the ferric heme iron. In contrast, when the ligand is nitrite, the spectrum indicates the formation of an intermediate that has spectroscopic and functional (reactivity with an N₂O₃ sensitive fluorescent probe) properties consistent with a recently proposed nitrite anhydrase reaction². In this mechanism, the NO reacts with the heme bound nitrite to form an N₂O₃ coordinated to a ferrous heme. This proposed intermediate is especially significant in that it represents a potential pathway for a nitrite-dependent catalytic process whereby Hb generates relatively long lived bioactive forms of NO such as S-nitrosoglutathione as well as the allosterically modulated β93 Cys-SNO. The failure to form this intermediate either at low pH or when the glass is extensively dried is consistent with the requirement for a specific conformation of reactants and residue side chains within the distal heme pocket. The role of the sulfhydryl groups from Cys β93 in this mechanism remains to be established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Diagram of the protocol used for exposing thin Hb containing glassy films to gaseous nitric oxide (gNO).

Figure 2.

Q band absorption spectrum of three different glass-embedded met Hbs as a function of time before and subsequent to the addition of gNO at time $t=0$ minutes. The labeled dotted reference lines indicate the peak positions for the NO metHb derivative (NO-Fe³⁺Hb). Unless otherwise noted all samples are prepared from a stock solution at pH 7.5.

Figure 3.

Q band absorption spectrum of glass-embedded aquomet Hb (Panel a) and nitrite metHb (Panel b) as a function of time before and subsequent to the addition of gNO at time $t=0$ minutes. The pairs of dotted lines at 535 and 565 nm, 543 and 573 nm, 538 and 567 nm indicate the approximate peak positions for NO metHb, NO ferrousHb and nitrite metHb. These glassy samples were allowed to dry for a longer period than for the samples associated with Fig. 2.

Figure 4.

The pH dependence of the Q band absorption spectrum of glass-embedded nitrite metHb as a function of time before and subsequent to the addition of gNO at time $t=0$ minutes. Panels a and b correspond to samples prepared at pH 6.5 and 7.5, respectively.

Figure 5.

The Q band absorption spectrum of glass-embedded nitrite methHb (Panels a and b) and nitrate methHb (Panel c) as a function of time before and subsequent to the addition of gNO at time $t=0$ minutes. The samples giving rise to the spectra shown in Panels b and c were dried for a much longer period than the sample associated with Panel a.

Figure 6.

The Q band absorption spectrum of a very dry glass-embedded nitrite methHb sample as a function of time before and subsequent to the addition of gNO at time $t=0$ minutes. The dotted lines show the peak positions for NO methHb.

Figure 7.

The Q band absorption spectrum of a very dry glass-embedded aquometHb sample as a function of time before and subsequent to the addition of gNO at time $t=0$ minutes. With the extreme drying the aquomet Hb spectrum evolved to the one shown which is characteristic of the Hb hemichrome (see text for details).

Figure 8.

Increase in fluorescence from an anaerobic DAF-2 solution (10 μ M, pH 7.5) exposed to glassy film containing a detectable level of “intermediate” as reflected in the absorption spectrum. The intermediate is accessed by exposing a thin glassy sample of the nitrite methHb derivative to gaseous NO under anaerobic conditions until the absorption spectrum shows indications of formation of the intermediate (see inset). The NO is then thoroughly flushed out and the glassy sample still under rigorous anaerobic conditions is then exposed to an aliquot of solution containing DAF-2 (see text for details). The control spectrum shown as the bottom fluorescence trace is from the DAF-2 solution prior to exposure to the glassy film and the sequence showing the increase in intensity is the progression as a function of time after the

DAF-2 solution is exposed to the glassy film (see text for details). Inset shows the initial absorption spectrum of the met Hb nitrite sample prior to exposure to gaseous NO under anaerobic conditions (in red) and the spectrum of the “intermediate” that occurs hours after the sample is exposed to NO under anaerobic conditions (in black). Not shown is the lack of response of the DAF fluorescence when, under anaerobic conditions, the DAF solution is exposed to: NO by itself, the starting glassy sample prior to introducing NO, and the sample after fully evolving to the ferrous NOHb derivative.