Metastable CO Binding Sites in the Photoproduct of a Novel Cooperative Dimeric Hemoglobin

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ABSTRACT The infrared absorption spectrum of the CO-photoproduct from *Scapharca inaequivalvis* hemoglobin (HbI) at 10 K yields only a single line in the "B" state region at 2132 cm^{-1}. This is the same frequency as the B_1 line observed in photodissociated vertebrate HbCO and MbCO. No evidence was found for the B_2 line detected in vertebrate hemoglobins and myoglobin in the 2118–2120 cm^{-1} region. These data demonstrate that the protein does not have the same conformationally accessible ligand-binding sites as do vertebrate hemoglobins and myoglobins. The absence of the B_2 line indicates that only a single weak site is accessible to the photolyzed CO molecule. These results are in accord with geminate rebinding experiments and ligand escape pathway calculations which have shown that the distal properties of HbI are distinct from those of tetrameric hemoglobins and vertebrate myoglobins.

INTRODUCTION

The cooperative dimeric hemoglobin (HbI) from the Arcid clam, *Scapharca inaequivalvis*, has many novel properties. It forms a dimer of identical subunits in contrast to vertebrate hemoglobins which form tetramers of distinguishable subunits (Chiancone et al., 1981). Furthermore, the hemes in the dimer are much closer to each other than they are in tetrameric hemoglobins (Royer et al., 1989; Chiancone et al., 1990). The interactions between the hemes have been studied by crystallographic (Royer et al., 1990) and spectroscopic methods (Spagnuolo et al., 1983; Antonini et al., 1984; Inubushi et al., 1988; Chiancone and Gibson, 1989; McGourty et al., 1989; Coletta et al., 1990; Chiancone et al., 1993; Rousseau et al., 1993; Song et al., 1993) in an effort to understand the molecular basis for cooperativity in this unique globin. Properties of the ligand binding site on the distal side of the heme have also been studied by these methods, as well as by photodissociation studies (Antonini et al., 1984; Chiancone and Gibson, 1989; Chiancone et al., 1983). In the photodissociation studies, it was found that the geminate recombination of O_2 and NO is more rapid in HbI than in myoglobin and also that these ligands escape from the heme vicinity more rapidly (Chiancone and Gibson, 1989). Moreover, modelling studies indicated that the pathway for ligand escape from the protein is very different in *Scapharca* hemoglobin than in myoglobin (Chiancone et al., 1993). These results demonstrate that there may be significant differences in the distal pocket of HbI as compared to myoglobin and vertebrate hemoglobins. However, the kinetic studies do not reveal the molecular basis for this difference and the modelling studies have not identified the number of possible metastable ligand binding sites in the protein.

In many hemoglobins and myoglobins the properties of the ligand binding pocket near the heme have been studied by examination of the metastable species formed after photodissociation of a bound ligand from the heme (Alben et al., 1982; Fiamingo et al., 1982; Dyer et al., 1989; Rothberg et al., 1990, 1992; Roberson, 1991; Anfinrud et al., 1989; Park, 1988). Most attention has focussed on CO, as this ligand may be studied by infrared absorption spectroscopy when it is bound to the iron atom and after photodissociation when it is loosely bound to residues in the heme pocket. Time resolved spectroscopy (Dyer et al., 1989; Rothberg et al., 1990, 1992; Roberson, 1991; Anfinrud et al., 1989) and cryogenic studies (Alben et al., 1982; Fiamingo et al., 1982; Park, 1988) have proven valuable in elucidating the geometric properties, e.g., the orientation of the ligand and the location of the metastable binding sites. One set of sites, designated as the A states, has been identified when the CO is bound to the iron atom of the heme, and another set, the B states, has been identified subsequent to photodissociation when the CO is loosely bound to amino acid residues of the protein. To determine the relationship between metastable ligand binding sites in HbI(CO) and in tetrameric human adult hemoglobin, HbA(CO), we obtained the infrared absorption spectrum of both of these proteins at cryogenic temperatures subsequent to photodissociation. We find differences in the metastable ligand rebinding sites of HbI which may play a role in its unusual geminate rebinding kinetics.

EXPERIMENTAL METHODS AND PROCEDURES

S. inaequivalvis HbI was isolated and purified according to established methods (Chiancone et al., 1981). Human hemoglobin was freshly prepared by the method of Rossi-Fanelli et al. (1958).

HbI samples for the infrared absorption measurements were prepared in 0.1 M phosphate buffer (pH 7.0) in 60% ethylene glycol at a heme concentration of 7 mM. They were deoxygenated, reduced with dithionite, exposed to CO, and then sealed in the infrared absorbance cell. The HbA

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samples were prepared the same way but the heme concentration was 8.5 mM. The absorbance cell had calcium fluoride windows which sandwiched the hemoglobin samples with a 50-μm spacer. It was attached to the cold finger of a Heli-Tran refrigerator that was cooled with liquid helium. Calibration of the temperature indicated that for a reading of 5 K on the cold finger, the sample temperature was about 10 K. The absorption spectra were obtained on a Nicolet 5DX FTIR instrument at 4 cm^{-1} resolution. To measure the frequencies of the CO stretching modes before and after photolysis, we first obtained the infrared absorption spectrum of CO-bound samples at ~10 K. Without moving the samples, they were illuminated with a 150-W white light through fiber optics for 10–30 min to generate the photoproduct. Typically, about 65% of the A-state absorption was bleached during the photolysis cycle.

RESULTS

The light minus dark infrared absorbance spectra at 10 K for both HbA and HbI are compared in Fig. 1. The insert gives an expanded region of the photoproduct. For HbA(CO) in the dark, the dominant peak (designated as A_1) is located at 1952 cm^{-1} with an associated ^{13}CO natural abundance isotope band at 1907 cm^{-1} (Alben et al., 1982). For both HbA and HbI only a single line in the A-state region is detected, at variance with the spectrum of myoglobin where a series of lines, A_0, A_1, A_2, and A_3, is found (Alben et al., 1982). No lines assignable as the A_0, A_2, or A_3 states are detected in the hemoglobins at cryogenic temperatures under our conditions.

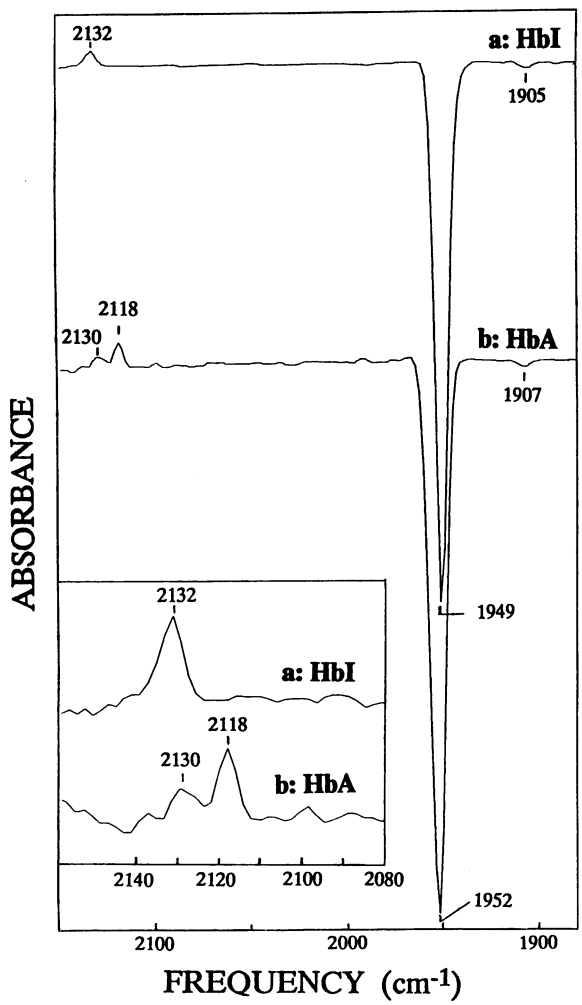

FIGURE 1 Infrared absorption difference spectra of photodissociated minus nonphotodissociated *S. inaequivalvis* hemoglobin (*a*) and human adult hemoglobin (*b*). The B-state region of the photoproducts is expanded in the insert. The data were obtained at 10 K and represent about 1 h of signal averaging for each state. The lines at 1905 and 1907 cm^{-1} result from the natural abundance ^{13}CO isotope.

Upon photodissociation of HbA(CO) two new bands appear at 2130 (B_1) and 2118 (B_2) cm^{-1} in the infrared difference spectrum, confirming the results of Alben and Park (Park, 1988). In unphotolyzed HbI(CO) the A_1 state is found at 1949 cm^{-1} similar to the room temperature value recently obtained by resonance Raman spectroscopy (Song et al., 1993). In the photoproduct only a single line is found, 2132 cm^{-1}, very close to the B_1 state in HbA(CO) (Park, 1988) and in Mb(CO) (2131 cm^{-1}) (Alben et al., 1982). If any contribution from a B_2-type state is present in the photodissociated sample, its intensity must be less than ~10% of that of the B_1 line. Moreover, no B_2 line from photodissociated HbI appeared in the 10–20 K temperature range which we investigated.

DISCUSSION

The low temperature infrared absorption spectrum of CO-bound HbI and CO-bound HbA contains only a single sharp band in the C—O stretching mode region of the Fe—C—O moiety. Lines in this region have been designated as "A-state" lines. For myoglobin the strong central line (termed the A_1 line) is flanked by side bands with A_0 appearing at higher frequency and A_2 and A_3, appearing at lower frequency. Essentially, only the A_1 line is present in the infrared spectrum of HbI and HbA at cryogenic temperatures. However, weak conformational substates that rapidly interconvert have been detected in a variety of hemoglobins in room temperature studies (Porter et al., 1990). Our data at cryogenic temperatures indicate that the bound CO has a more limited conformational subspace in both the tetrameric and dimeric hemoglobins than in myoglobin.

Photodissociation of carbon monoxide bound hemoglobins and myoglobins leads to states in which the CO is associated with amino acid pockets in the protein along its pathway for escape. Such states, termed the B states, have been characterized at both low and high temperatures (Alben et al., 1982; Fiamingo et al., 1982; Dyer et al., 1989; Rothberg et al., 1990, 1992; Anfinrud et al., 1989; Park, 1988). In HbA(CO) and Mb(CO) three B states have been identified: B_0 in which the CO coupling to the protein is weakest and the C—O stretching frequency is similar to that of the free CO frozen in an inert matrix (2144 cm^{-1}); the B_1 state with a C—O stretching frequency of 2130–2132 cm^{-1}; and the B_2 state with a C—O stretching frequency of 2118–2120 cm^{-1}. At low temperatures (below 10 K) the B_1 and B_2 lines are both present in the spectra of photodissociated vertebrate HbA(CO) and Mb(CO) (Alben et al., 1982; Park, 1988). As the temperature is increased, the population of the B_2 line

(2118 cm^{-1}) decreases, while the B_1 line (2130 cm^{-1}) increases. Finally, the B_1 line also starts to decrease as the CO-bound heme becomes fully repopulated. In addition, in HbA(CO) at 10 K with continuous illumination, the B_1 state is optically pumped into the B_2 state.

The importance of the B states in the functional properties of hemoglobins and myoglobins has been pointed out several times in the past when ligand recombination to the iron and ligand escape from the protein were being considered (Alben et al., 1982; Hofrichter et al., 1983; Ansari et al., 1986; Chiancone and Gibson, 1989). In the simplest case sequential models of the general type,

$$A \rightleftharpoons B \rightleftharpoons F,$$

have been proposed. In this model A represents states in which the ligand (CO) is bound to the heme; B represents states in which it is dissociated from the heme but loosely bound to residues in the protein; and F represents states in which the ligand is no longer associated with the protein. The properties of these states and the kinetic barriers between them determine the ligand rebinding and escape properties of the globin.

At room temperature this simplified scheme is complicated by conformational changes of the protein subsequent to ligand release from the heme (Hofrichter et al., 1983; Agmon and Hopfield, 1983; Ansari et al., 1985). Thus, the barriers for transitions among these states are dynamic functions of time and temperature. Such changes have been clearly identified in the dynamics of CO photodissociation from *S. inaequivalvis* (Chiancone and Gibson, 1989; Chiancone et al., 1993). Therefore, analysis of the B state at cryogenic temperatures offers a means to study the intermediate states of the CO bound to the protein in the absence of the protein conformational changes that take place at higher temperatures. Such intermediate configurations do exist, however, at physiological temperatures in hemoglobin and myoglobin (Rothberg et al., 1990, 1992; Roberson, 1991; Anfinrud et al., 1989) and have been demonstrated to be relevant to the pathway by which ligands enter and leave the heme proteins.

In the data on photodissociated HbI(CO) only a single line is found with a frequency (2132 cm^{-1}) placing it as a B_1 state. Within our signal-to-noise ratio there is no evidence for the presence of any contribution from a B_2 state unlike both HbA(CO) and Mb(CO) (Alben et al., 1982; Park, 1988). We are unable to relate the cryogenic temperature studies quantitatively to the properties at physiological temperatures. However, some qualitative conclusions may be drawn. The presence of two distinct B states in tetrameric hemoglobins and in myoglobin in contrast to the presence of only one B state in HbI indicates that the properties in the distal pocket and hence the pathway for ligand escape from the protein is different in HbI as compared to the other globins. The absence of a B_2 state in HbI indicates that either the protein conformation does not allow for the formation of such a state or, once formed, the barriers for its relaxation are too low to allow for its detection under our conditions.

Recently, the geminate rebinding of O_2, NO, and CO following ligand-photodissociation in HbI have been reported and found to have several unique features (Chiancone and Gibson, 1989; Chiancone et al., 1993). First, after photodissociation, as compared to myoglobin, NO and O_2 rebind more rapidly with the heme and escape from the pocket more rapidly. Second, analysis of the ligand rebinding and escape data implicated different populations of ligand-protein associated states (B states) in HbI as compared to myoglobin. Third, O_2, NO, and CO share a common pathway for diffusion to the exterior. Fourth, modelling studies suggest that the pathway for ligand diffusion does not follow the classical myoglobin pathway through the histidine-valine gate (Case and Karplus, 1979). These differences indicate that the distal environment in *S. inaequivalvis* is unique. Our observation that only one B-type binding site is present in HbI is additional evidence that the structural properties of the distal pocket are not the same as those of tetrameric hemoglobin and myoglobin. At low temperature, HbI has only a single shallow well available for binding the photodissociated ligand in contrast to vertebrate hemoglobin and myoglobin in which two wells are accessible. The absence of the second protein-ligand bound state in HbI could contribute to the unique geminate properties of the hemoglobin. It appears that the photodissociated ligand interacts more strongly with the protein in vertebrate hemoglobin and myoglobin than in HbI. Thus, in HbI, as compared to HbA, the distal pocket plays a less dominant role in controlling the ligand rebinding and escape from the protein.

Finally, it should be emphasized that the inferences drawn in the present study are based on the cryogenic experiments. To relate photodissociation measurements properly to functional behavior at physiological conditions, time-resolved measurements at room temperature must be made. Furthermore, computer modelling and infrared dichroism studies have led to the identification of the two specific ligand-protein binding sites in vertebrate hemoglobin (Roberson, 1991; Rothberg et al., 1992). Similar studies will be carried out in HbI to determine if the single B state in HbI is the same as the B_1 state of HbA or is a completely different type of site.

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