Demonstration of intermediates between deoxy and carbonmonoxy hemoglobins by anaerobic high-performance liquid chromatography

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Intermediary ferrous hemoglobins (Hb) partially liganded with carbon monoxide (CO) were separated using an anaerobic high-performance liquid chromatography system equipped with a cation-exchange column at room temperature. Separations were achieved by applying various mixtures of oxy- and CO-Hb A to the column. The mobile phase contained 3 mM sodium dithionite which deoxygenated the oxy hemes but left the CO hemes intact, thereby producing hemoglobins bound with various numbers of CO molecules. Spectrophotometric analyses of the eluent showed that hemoglobins were eluted in the order of deoxy hemoglobin, Hb(CO), $Hb(CO)_2$, $Hb(CO)_3$ and $Hb(CO)_4$.

Hybrid hemoglobin *Partially liganded hemoglobin* *HPLC*

1. INTRODUCTION

Although the detailed structures of liganded and unliganded hemoglobins (Hb) are well known, the structures of partially liganded hemoglobins have not been elucidated. Hemoglobins exist in vivo in a partially saturated state. A complete understanding of the structure-function relationship in hemoglobin, especially allostery, requires knowledge of the structural and functional properties of partially liganded hemoglobins. Perrella et al. [1,2] successfully separated intermediates between met- and CO-saturated hemoglobins using isoelectric focusing. However, with this method, it was necessary for the unliganded hemes to be in the met form and for experiments to be done at subzero temperatures.

Recently, we found that asymmetrical hybrid hemoglobins, formed in a mixture of two hemoglobin species, can be separated by anaerobic ion-exchange high-performance liquid chromatography (HPLC) at room temperature [3,4]. Since liganded and unliganded hemoglobins have different quaternary structures, it is conceivable that they, along with any sufficiently stable partially liganded tetramers, could also be separated by ion-exchange HPLC. We report using an anaerobic HPLC system for the successful separation of intermediate forms of ferrous hemoglobin A bound with one to four CO molecules.

2. EXPERIMENTAL

Analyses by HPLC were done using a Waters Associates system consisting of two model 6000-A pumps, a model 450 variable wavelength detector, a U6K injector, a data module, and a 720 system controller. For most experiments, the chart speed was 18 cm/h, and the detector was set at 424 nm (the isosbestic point of CO-Hb and deoxy-Hb) with a full-scale absorbance of 0.1. Spectrophotometric measurements were done using a

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Varian Cary 2300 spectrophotometer. The eluting species were collected in ordinary cuvettes under paraffin oil to insure isolation from air, and their absorbance spectra were determined between 380 and 460 nm. The fraction of CO-bound hemes present in the cuvette was calculated according to the equation

$$\text{CO-Hb A (\%)} = \frac{(A_{419}/A_{424}) - 0.82}{0.72} \times 100$$

where A_{419} and A_{424} represent the absorbance at 419 and at 424 nm, respectively.

Hb A was purified from Hb AS blood by column chromatography on DEAE-Sephadex [5]. Samples were prepared in a buffer equal in molarity to, and slightly higher in pH, than the initial buffer used in HPLC separations. The fraction of CO-bound hemes in the samples was measured spectrophotometrically. All hemoglobins were free of organic phosphates. All samples were filtered before being applied to the column. Approx. 20 μg Hb were used for each experiment.

Separations were done using a cation-exchange TSK gel CM-3SW column (7.5 × 150 mm) kindly provided by Toyo Soda, Tokyo. Elution was achieved with a gradient of two buffers. The initial buffer (buffer I) consisted of 25 mM potassium phosphate, 3 mM $Na_2S_2O_4$ (sodium dithionite), and 0.5 mM EDTA, pH 6.5. The final buffer consisted of 50 mM potassium phosphate, 3 mM $Na_2S_2O_4$ and 0.5 mM EDTA, pH 7.4. The flow rate was 0.8 ml/min. The separations were achieved as follows: 100% I, 10 min; 100–90% I, 10 min, curve 6; 90–50% I, 15 min, curve 6; 50–0% I, 15 min, curve 6; 0% I, 5 min.

Buffers were filtered and sonicated before use, and separations were carried out at room temperature. Care was taken to adjust accurately the pH of the buffers, since small changes in pH have a considerable effect on resolution and elution time.

3. RESULTS AND DISCUSSION

When a mixture of two species of liganded hemoglobin is chromatogrammed in the absence of sodium dithionite, the elution pattern exhibits two peaks corresponding to the two hemoglobins. In fact, this can be considered an artifact since such a mixture actually contains three components: the two parent hemoglobins and a hybrid form [6–8]. Such a hybrid hemoglobin cannot be separated by routine chromatographic techniques because liganded hybrid hemoglobin dissociates quickly into dimers that reassociate with like dimers to reform the parent hemoglobins. We recently found that if two species of hemoglobin were mixed in the oxy form and chromatogrammed using HPLC under anaerobic conditions, a peak representing the hybrid hemoglobin could be demonstrated in addition to the two peaks of the parent hemoglobins [3,4]. For these separations, the oxyhemoglobins were instantly deoxygenated by sodium dithionite which was previously added to the mobile phase, thereby fixing all hemoglobins in the relatively stable 'T' quaternary conformation.

Assuming that this technique could be used to separate hemoglobins in which 0–4 ligands are bound to their hemes, various mixtures of oxy- and CO-Hb A were applied to a cation-exchange HPLC column under anaerobic conditions. These mixtures were assumed to contain various forms of hemoglobin owing to the exchange of oxygen and carbon monoxide and to the formation of hybrid hemoglobins. Upon application of the mixture to the column, the oxy hemes would be deoxygenated by dithionite, while the CO hemes would be unaffected. Since the exchange rate of carbon monoxide is relatively slow, the mixture should contain 5 species of hemoglobin, with carbon monoxide being bound to 0–4 hemes. The fraction of each species in the mixture would depend on the initial percentage of CO saturation.

Results for fully deoxygenated and fully saturated CO forms of Hb A which were eluted under the same buffer and pH conditions are shown in fig.1a,e. Deoxy-Hb A and CO-Hb A eluted about 8 min apart, apparently because of the difference in quaternary structure between liganded (R conformation) and unliganded (T conformation) hemoglobins.

We applied different mixtures of oxy- and CO-Hb A to the column. We collected the eluted species represented by the relatively distinct peaks in the chromatograms and determined spectrophotometrically the percentage of CO-bound hemes. We found that hemoglobins with a higher number of CO-bound hemes were slower to elute.

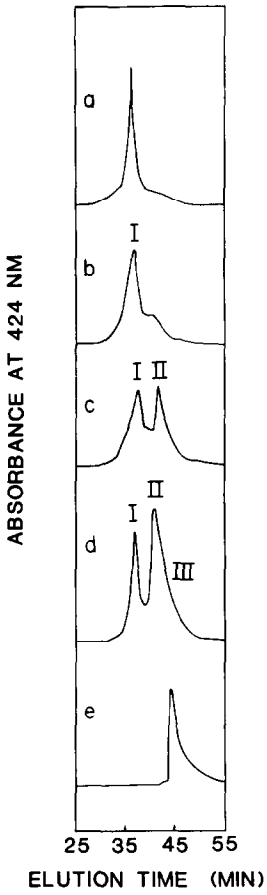

Fig.1. Separation of partially liganded hemoglobin species by anaerobic HPLC. Conditions for separation are outlined in section 2. (a) Deoxy-Hb A; (b) a 30:70 mixture of CO- and oxy-Hb A; (c) a 46:54 mixture of CO- and oxy-Hb A; (d) a 72:28 mixture of CO- and oxy-Hb A; (e) fully CO-saturated Hb A. The oxy hemes were deoxygenated by dithionite upon application to the column.

Fig.1b shows the chromatogram of a 30:70 mixture of CO-Hb A and oxy-Hb A. Spectrophotometric analysis showed that the percentage of CO-Hb in the main peak (peak I) was 22%. Fig.1c shows the chromatogram of a 46:54 mixture of CO-Hb A and oxy-Hb A. The chromatogram shows two main peaks, peak I (19% CO-Hb) and peak II (46% CO-Hb). The chromatogram of a 72:28 mixture of CO-Hb A and oxy-Hb A also contains two main peaks, peak I and peak II (fig.1d). The fractions of CO hemoglobin in those peaks were 27 and 52%, respectively. The hemoglobin obtained in the shoulder area (III) following peak II had a CO saturation of 79%. It is noteworthy that in all runs, the overall percentage of CO-Hb A detected was slightly lower than that of the original sample. This decrease can be attributed to the loss of CO into the liquid phase.

The relationship between the percentage of CO hemes in the collected peaks and their approximate elution times in several of our experiments is shown in table 1. Partially liganded hemoglobins were eluted at positions intermediate between those of fully deoxy-Hb A and fully saturated CO-Hb A. Since the resolution was not sufficient, the measured percentage of CO hemes in collected peaks sometimes reflected some overlapping and fluctuation, depending on the specific part of the peak that was collected. Nevertheless, the results demonstrate that hemoglobin species partially liganded with CO could be detected by anaerobic HPLC. In fig.1, peaks Ib–Id may represent Hb(CO). Peaks IIc and IId may represent $Hb(CO)_2$. Shoulder IIId may contain $Hb(CO)_3$.

We did not attempt to stabilize the isolated partially liganded hemoglobins. Indeed, these forms, after being collected in cuvettes, will reequilibrate rather quickly to form a mixture of hemoglobins with different levels of ligation. Nevertheless, the average fraction of CO-bound hemes in the cuvettes represent the fraction of CO in the collected peak. Reequilibration in the column would produce different forms of partially liganded hemoglobins with different migration speeds

Table 1

The relationship between the percentage of CO in collected peaks and their approximate elution time

Approximate time (min)	Measured CO in collected peak (%)	Number of CO molecules bound to Hb tetramers
36	0	0
37	19–27	1
40	38–54	2
42	71–79	3
44	100	4

Experimental conditions are described in section 2. Results for 0 and 100% CO-Hb were obtained using completely deoxy- and CO-hemoglobins, respectively

related to the level of ligation. Hemoglobin molecules of each form would assemble to produce a single chromatographic peak. The isolated forms are apparently stable enough to survive detection and collection.

The basis for the separation of partially liganded ferrous hemoglobins is unknown. However, separation is not due to a charge difference in the heme iron as was the case when compounds intermediate between ferric and CO hemoglobin were separated by Perrella et al. [1,2], nor is the separation due to the R-T conversion in hemoglobin. It is more likely that the separation is due to differences in quaternary structure among partially liganded ferrous hemoglobins, differences evident from the position of elution of these forms relative to the elution positions of deoxy-Hb A and $Hb(CO)_4$ A. The structure may depend on the hemoglobin molecule's level of ligation.

Our results show the transient isolation of ferrous hemoglobins partially liganded with CO. The HPLC method can be used to clarify the structure-function relationship of partially liganded hemoglobin, but a means must be found to fix partially liganded hemoglobins at a particular ligation level.

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