Heme-CO as a probe of the conformational state of calmodulin

M.C. Marden, L. Leclerc and C. Poyart

INSERM U299, Hôpital de Bicêtre, 94275 Le Kremlin Bicêtre, France

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The interaction of heme-CO with calmodulin, in the presence of calcium, leads to a complex of four heme-CO molecules per protein. No interaction was observed in the absence of calcium. The binding of heme-CO to calmodulin was monitored by the shift in the Soret absorption band from 407 to 420 nm (bound form); the four sites are not spectrally identical. The ligand CO can be photodissociated from the calmodulin-heme-CO complex and the bimolecular recombination kinetics also indicate a heterogeneous mixture. The complex does not bind oxygen reversibly. As calmodulin has only one histidine, the hemes are apparently not bound by the iron atom as in hemoglobin, but are probably loosely associated ($K_d = 0.5$ μM) in hydrophobic pockets which apparently open when the protein is activated by calcium.

Heme; Calmodulin; Calcium; Protein conformation

1. INTRODUCTION

The binding of iron protoporphyrin IX (heme) to its natural globins of hemoglobin (Hb) and myoglobin (Mb) occurs at a very high affinity (pM) [1]. Other proteins are known to bind heme at moderately high affinity: hemopexin contains a single site of nM affinity, in order to remove any excess free heme [2]. Serum albumin, also present in the plasma, contains one site in the nM range [2–5] and additional sites of lower affinity. There appears to be an entire class of proteins that bind heme at the μM range. Spectrin, the major component in the red blood cell cytoskeleton, has been observed to bind over 10 hemin molecules [6–8]. Several other proteins have been investigated, such as the red blood cell proteins 4.1 and actin [9] and found to have similar properties. In the presence of hemin, the activity of the calmodulin-ATPase system of the red blood cell membrane is inhibited [10].

Thus heme may be involved in other reactions in addition to its primary functions of oxygen transport and electron transfer. Most heme binding studies have employed the ferric form hemin. The ferrous heme-CO also binds to many proteins and permits ligand binding measurements [5]. Heme binding studies may serve to probe the surface or hydrophobic pockets of proteins, and as a probe of conformational changes in the protein which expose new binding sites.

2. MATERIALS AND METHODS

Hemin (Sigma) was dissolved in 0.1 M NaOH and subsequently diluted in 50 mM Tris buffer at pH 7.4. Fresh solutions were prepared to avoid aggregation of the hemes. The hemes were reduced by addition of a buffered solution of Na-dithionite under an atmosphere of CO. The heme-CO concentration was calculated from the maximum Soret (407 nm) absorption with $\epsilon = 147$ (per mM and per cm) [1]. Static absorption spectra were measured with a Cary 219 (Varian) spectrophotometer.

Calmodulin from hog brain and two inhibitors, trifluoperazin (M_w 440.4) and calmidazolium (M_w 687.7) were products of Boehringer. Calmodulin stock concentration was determined using the UV band at 276 nm with $\epsilon = 3740$ (per M per cm) [11]. Because commercial calmodulin contains some residual calcium, ethylene-glycol bis(2-aminoethylether)-*N,N'*-tetraacetic acid (EGTA, Serva) was used for the measurements without calcium.

Cosolvents were used to change the solvent hydrophobicity: ethanol and glycerol (Prolabo), ethylene glycol (Baker), and dimethyl sulfoxide (DMSO from Sigma). The free heme-CO Soret peak is at 413 nm in 50% DMSO/water solutions.

CO recombination kinetics were measured after photodissociation by a 10 ns pulse at 532 nm (Quantel YG 585). Samples were in 1 mm optical cuvettes with 60 μM heme and 20 μM calmodulin.

3. RESULTS

The visible absorption spectra for heme-CO and for the complex [calmodulin-calcium] · [heme-CO], in 50 mM Tris buffer at pH 7.4, are shown in Fig. 1. The Soret band for heme-CO is shifted from 407 nm to about 419 nm, with a decrease in the absorption coefficient and an increase in the half-width for the Soret band. The peaks at 540 and 576 nm become more equal in amplitude. Unlike Hb or Mb, oxygen rapidly produces the ferric species hemin. No interaction, based on the absorption spectra, was observed for hemin with either form of calmodulin.

Without calcium (with 100 μM of EGTA), the fraction of heme-CO bound to calmodulin was less than 5%. Addition of calcium (200 μM) could then provoke the heme-CO binding reaction. Finally, if EGTA was added to the bound complex (over 200 μM to remove

Correspondence address: M.C. Marden, INSERM U299, Hôpital de Bicêtre, 94275 Le Kremlin Bicêtre, France

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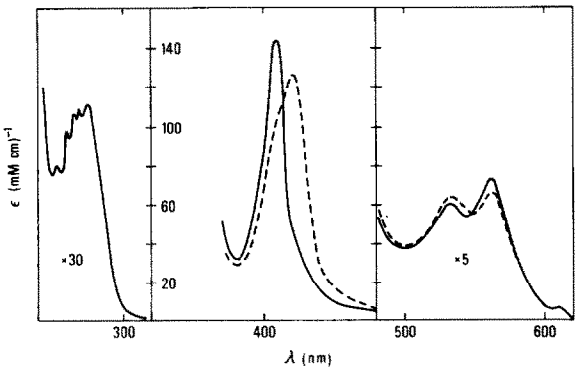

Fig. 1. Absorption spectrum of calmodulin (——), heme-CO (——), and the complex [calmodulin-calcium] · [heme-CO] (– – –) at 25°C in 50 mM Tris buffer, pH 7.4, 200 μM calcium, 10 μM heme, and 3 μM calmodulin.

the calcium), the free heme-CO spectrum was recovered.

Addition of cosolvents such as ethanol, DMSO, glycerol, or ethylene glycol to the bound complex shifts the spectrum towards the free heme-CO form. For [calmodulin-calcium] · [heme-CO], addition of ethanol to a final concentration of 20% ethanol by volume was sufficient to obtain the spectrum for heme-CO without protein.

Two inhibitors of calmodulin, trifluoperazin and calmidazolium, were tested to see if they compete with heme-CO for the same binding site. No conclusions can be drawn, as both of these hydrophobic molecules also interact with heme-CO.

Flash photolysis of [calmodulin-calcium] · [heme-CO] leads to biphasic CO recombination kinetics (Fig. 2), as previously reported for the BSA · heme-CO complex [5]. The kinetic difference spectra show two spectrally distinct components which are similar in amplitude. The faster component has a difference spectrum red shifted by a few nm relative to a control sample of heme-CO. The slower phase is further red shifted, being similar to Mb.

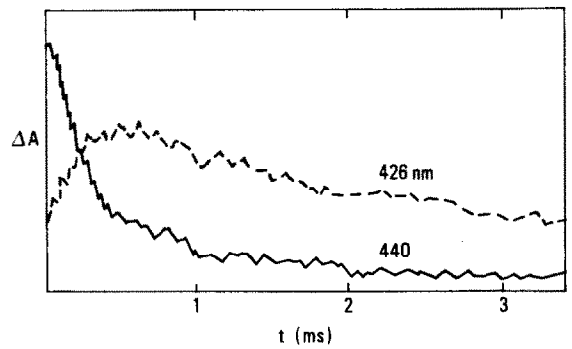

Fig. 2. Recombination kinetics of CO for the complex [calmodulin-calcium] · [heme-CO] afer photodissociation at 25°C, in pH 7.4 Tris buffer at the detection wavelength indicated.

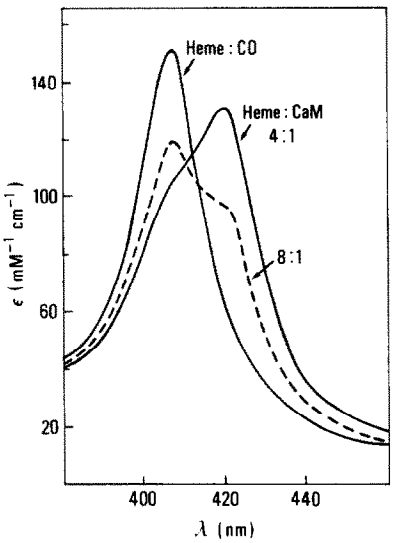

Fig. 3. Absorption spectra of heme-CO (——), and heme-CO with calmodulin (CaM) at a molar ratio of 8:1 (– –) and 4:1 (——). Conditions: 10 μM heme, 200 μM calcium, Tris buffer, pH 7.4, 25°C.

The stoichiometry of the binding of heme-CO to protein was measured by preparing samples with excess heme relative to the protein and calculating the fraction of heme bound from the absorption spectra. Spectra in the Soret region are shown in Fig. 3.

Addition of calcium bound calmodullin showed that four molecules of heme-CO can bind. Analysis near the peak for the bound form (near 420 nm) gave results of 4.1 ± 0.1. However, the analysis was wavelength dependent, indicating that the spectra for the four bound hemes are not the same. Two spectral forms would also explain why the Soret peak is broader and less intense for the bound form relative to free heme-CO. At higher protein concentrations (heme-CO to protein ratios below 2), the Soret peak is better defined as a single absorption band.

Since the four sites are not identical, it is difficult to determine whether the heme binding occurs in a cooperative fashion, as suggested for the binding of calcium [12]. We therefore only report an average dissociation constant of 0.5 μM.

4. DISCUSSION

The results indicate that four molecules of heme-CO bind to calmodulin when activated by calcium, but no interaction occurs for the inactive calcium free conformation. The static and kinetic spectral measurements both suggest four non-identical heme binding sites, with perhaps two distinct populations.

It is of course of interest to locate the binding sites. As there is only one histidine residue, we can eliminate iron-histidine bonds as in Hb or Mb. The cosolvent studies also show the reaction does not involve a strong chemical bond, but rather a hydrophobic interaction.

There are two possibilities suggested from the X-ray studies.

The four heme-CO binding sites might reflect the symmetry of calmodulin for its natural effector–calcium. X-ray crystallographic results show a close similarity in the four calcium binding domains [13]. While heme may be too large for these sites, it might share part of the hydrophobic pocket; large ligands bind to Mb and Hb [14], although there may not be any evident pocket from the static structure.

A second possibility for the heme-CO binding sites are the two large hydrophobic clefts in calcium bound calmodulin, described by Babu et al. [13]. The surface of each cleft is approximately 10×12 Å^2, which could accommodate two heme molecules. This might also explain why the spectral changes are not identical for the four hemes, since the first heme would see an empty cleft and the second would require a rearrangement. The structure for the form without calcium is not known; the present solution studies suggest that calcium opens the clefts in calmodulin for its natural interaction with target proteins.

In general, a distribution of sites of various affinities must be considered. There are no heme-CO sites for calmodulin at the pM level as for Hb or Mb. At the 0.1–1 μM range, we have observed sites for nearly all proteins studied, including secondary sites for Mb and Hb. There are probably additional sites at even weaker concentrations, but these will be too non-specific to be useful probes.

The present observations are similar to those for studies of the binding of fluorescent probes to calmodulin. The binding of 9-anthroylcholine was calcium dependent and showed approximately four sites of unequal affinity [16]; the average dissociation constant of 200 μM being over 100 times larger than for the binding of heme-CO to calmodulin.

There are at least three applications for the heme·protein binding reactions. (i) The probing of hydrophobic pockets; the presence of these specific hydrophobic pockets may indicate some function, as in hemopexin and BSA for the removal of excess heme [2]. These pockets or hydrophobic surfaces might be involved in protein-protein interactions [15]; the interactions with heme then serve to probe the relative number and affinity of these sites.

(ii) A second application is to add color to otherwise white proteins. This permits spectral dosage in a visible region, useful in the case of calmodulin which has no tryptophans making UV dosage difficult. Since the heme group can quench the fluorescence of tryptophan and other fluorescent molecules, these artificial heme proteins could be used for studying protein-protein interactions or as an internal probe where the tryptophan-heme distances can be calculated.

(iii) A third potential application is as a probe of conformational changes in proteins. Heme-CO binding will be sensitive to structural changes which expose additional sites. The present example of calmodulin is ideal since there are no sites for the inactive form (without calcium); the heme binding signal is then an easily detected all or nothing reaction.

CONCLUSIONS

The results presented here led to the following conclusions: heme-CO binds to the active (calcium bound) form of calmodulin. The fact that calmodulin, which has only one histidine, binds four molecules of heme-CO implies that histidine is not essential and that the iron atom may not be bound as in Hb. The release of the heme-CO upon addition of cosolvents indicates that the interaction is hydrophobic in nature. A transition in calmodulin from a closed to an open structure occurs upon addition of calcium; heme-CO binds only to the open structure and may serve as a conformational probe.

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