CORRELATION BETWEEN THE QUANTUM YIELDS OF PHOTODISSOCIATION AND C–O STRETCHING FREQUENCIES OF CARBON MONOXIDE HEMOPROTEINS

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Received 4 December 1978

1. Introduction

Upon illumination, carbon monoxide (CO) is dissociated from the CO complex of reduced hemoproteins. Yields for this photochemical reaction are not the same among all hemoproteins; MbCO is highly sensitive to light [1], HbCO is less sensitive than MbCO [1,2] and carbon monoxide cytochrome *P*-450 of liver microsome is fairly insensitive [3]. Therefore, it is conceivable that the quantum yield depends upon the nature of an apoprotein moiety, which influences the electronic and chemical structures of the heme and its environment.

In an attempt to obtain information about the structures of heme vicinities responsible for the quantum yield alteration, we measured the quantum yield of $P450_{cam}$–CO as well as those of the CO complexes of other hemoproteins such as Mb and Hb under various conditions. We also measured infrared C–O stretching frequency (ν_{CO}) of the CO complexes, because it is known to be sensitive to environmental variations around heme.

Our results indicate that the quantum yield is influenced by a number of surrounding factors of the heme and that this influence is reflected in a relationship between the quantum yield and the ν_{CO}; the quantum yield decreases as ν_{CO} shifts toward higher frequency. A possible mechanism for this phenomenon is discussed.

Abbreviation: Mb and MbCO, myoglobin and its carbon monoxide complex; Hb and HbCO, hemoglobin and its carbon monoxide complex; $P450_{cam}$ and $P450_{cam}$–CO, cytochrome *P*-450 for D-camphor monooxygenase of *Pseudomonas putida* and its carbon monoxide complex; HRP, horseradish peroxidase; Pd, putidaredoxin

2. Materials and methods

2.1. *Enzymes and chemical reagents*

$P450_{cam}$ was purified from cell-free extracts of *Pseudomonas putida* [ATCC 17453] cultivated in the presence of D-camphor by the method in [4] with slight modifications [5]. The A_{392} A_{280} ratio of our purified enzyme was 1.26. Mb was purchased from Sigma and was used after a chromatography on a CM-cellulose (CM-52, Whatman). Hb was purified from fresh erythrocytes of adult human. HRP of B+C-type was purified by the method in [6]. An inactive form of $P450_{cam}$, P420, was prepared either by acetone treatment [7] or by allowing $P450_{cam}$ to stand overnight in the absence of D-camphor at room temperature. Other chemicals were of analytical grade from Nakarai Chemicals, Japan.

2.2. *Flash Photolysis*

Flash photolysis was performed using a pulse flash spectrophotometer (Union Giken RA 403) equipped with a kinetic data processer (System 71). An Xe bulb (100–J) with ~30 μs flash duration was used as a source of the exciting light. The photodissociation of samples was measured either by monitoring ΔA at a fixed wavelength or by recording the spectra of transient species with a double flash apparatus. The double flash apparatus was equipped with a multichannel photodiode for a detector and a sub-flash bulb (3 J) for a monitoring light source. The inten-

sity of the exciting light (flash) was varied with the aid of neutral density filters (Hoya Corp.) and the relative intensities were expressed in % by taking the intensity without a filter as 100%. The reaction cell was placed in a thermostated cell holder and the reaction was performed at 22°C throughout this study. CO complexes of hemoproteins were prepared by an addition of (1 atm) CO-saturated water to the sample reduced with sodium dithionite under nitrogen atmosphere. CO concentrations of the CO-saturated water was determined spectrophotometrically by titrating a solution of reduced Mb with the CO-saturated water.

2.3. *Infrared spectroscopy*

Fourier transform infrared spectrum of the CO form of hemoproteins was obtained by ratioing against the oxidized form using a JEOL 03F-FTIR spectrophotometer at 2 cm^{-1} resolution at room temperature. CO complexes of hemoproteins were prepared by reducing them with sodium dithionite under CO atmosphere (1 atm) and transferred into a cell with As_2Se_3 window with 0.1 mm light pathlength. The details will be described in [8].

3. Results and discussion

Since the quantum yield for MbCO has been taken as 1 [1,2], the photodissociation of MbCO was measured at pH 7.4 to obtain a standard for the measurement of the quantum yields for other hemoproteins. Figure 1A shows spectra of Mb recorded at 30 μs after MbCO was irradiated with the varying intensities of the flash. A family of spectra, which has a clear isosbestic point at 430 nm, indicates that MbCO was converted into the reduced form by the flash and that the amount of the conversion increased as flash intensities increased. When the intensity of the flash was raised to 20% of the full intensity, the spectrum obtained was indistinguishable from that of the reduced Mb indicating that all the CO form was photodissociated. In fig.1B, the % dissociation was plotted against the flash intensity showing a hyperbolic relationship between them. The % dissociation of various other hemoproteins was also found to have a hyperbolic relation with the flash intensity. Here, the hyperbolic curve is characterizied by $I_{1/2}$,

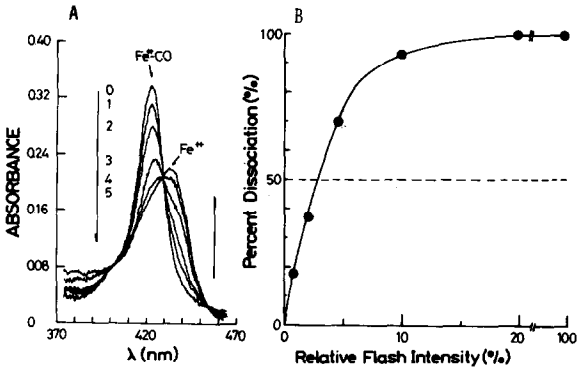

Fig.1. Effect of varying flash intensity on the photodissociation of MbCO. (A) The spectra were recorded at 30 μs after firing the flash. The relative flash intensities from 0–5 were 0, 0.9%, 2%, 4.5%, 10% and 20% of the full intensity, respectively. Reaction medium contained 1.9 μM Mb, 14 μM CO in 0.1 M potassium phosphate buffer (pH 7.4) at 22°C. (B) % dissociation was plotted against the relative flash intensity. Data were taken from (A).

which is the flash intensity at the 50% photodissociation. Since light quanta required for the photodissociation of a half amount of the CO-form are proportional to the product of $I_{1/2}$ and the extinction coefficient (ϵ) of the CO form at Soret band, we compared the $I_{1/2} \cdot \epsilon$ of various hemoproteins with that of Mb for the measurement of the quantum yield, which is the ratio of mol CO reacted to light quanta absorbed by the CO complex [1].

$P450_{cam}$, which has the camphor binding site besides heme, changes its electronic and chemical structures of the heme and its vicinity upon camphor binding [4,9]. The alteration of the quantum yield is expected from this structural change. Thus, the photodissociation of $P450_{cam}$–CO both in the presence and absence of D-camphor was measured with the experimental procedures above. Spectra of $P450_{cam}$–CO after the illumination of the flash of various intensities are illustrated in fig.2A and the % dissociation was plotted against the relative flash intensity (fig.2B). $I_{1/2}$ for $P450_{cam}$–CO, thus obtained, was 3.4% and 56.5% in the presence and absence of D-camphor, respectively. Since the absorption spectra of $P450_{cam}$–CO in the presence and absence of D-camphor are almost indistinguishable, the above difference in $I_{1/2}$ is reflected in the

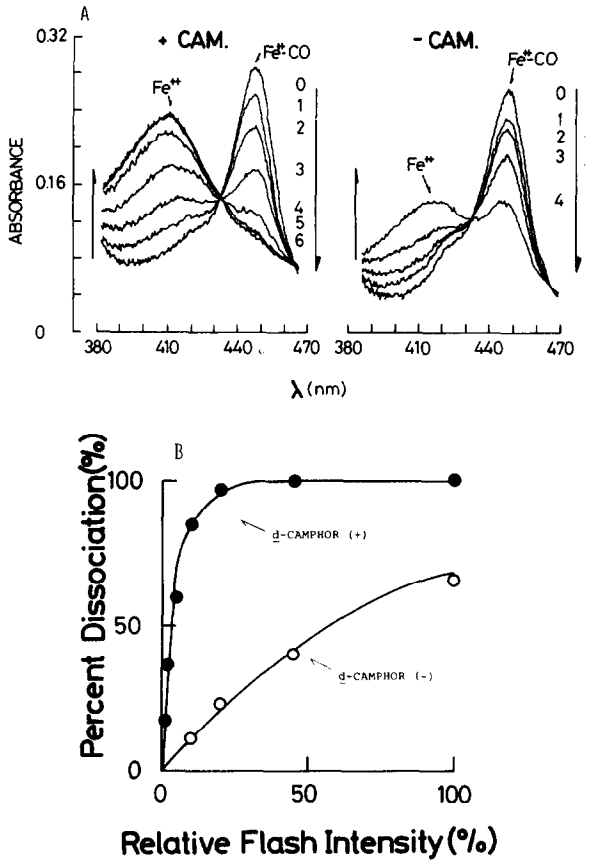

Fig.2. Effect of varying flash intensity on the photodissociation of $P450_{cam}$–CO. (A) In the presence of D-camphor (+ CAM), the relative flash intensities from 0–6 were 0, 0.9%, 2%, 4.5%, 10%, 20% and 45% of the full intensities, respectively. Reaction medium contained 3.1 μM $P450_{cam}$, 37 μM CO and 37 μM D-camphor. In the absence of D-camphor (– CAM), the relative flash intensities from 0–4 were 10%, 20%, 45% and 100% of the full intensity, respectively. Reaction medium contained 2.6 μM $P450_{cam}$ and 4.3 μM CO. Other details of the reaction conditions were described in the legend to fig.1. (B) % dissociation was plotted against the relative flash intensity. Data were taken from (A).

quantum yields, which were 1.0 and 0.06 in the presence and absence of D-camphor, respectively (table 1). In either case the quantum yield was not altered by changing pH from 6.6 to 8.8 and by changing KCl from 0 to 0.26 M in 0.1 M potassium phosphate buffer (pH 7.4). Upon addition of putidaredoxin (Pd), however, the quantum yield in

Table 1
Quantum yield for the photodissociation of CO complexes of various hemoproteins and their CO stretching frequencies

CO complex	Quantum yield (Φ)	CO stretching frequency (cm^{-1})
$P450_{cam}$ (pH 7.4)		
D-camphor (+)	1[a]	1940[a]
D-camphor (+) Pd[g] (+)	0.86[a]	
D-camphor (–)	0.06[a]	1959[a]
α-picoline[h] (+)	0.06[a]	1955[a]
P420 (pH 7.4)	0.03[a]	1965[a]
Mb (pH 7.4)	1[b]	1944[a,d]
HRP (pH 11)	1[a]	~1931[e]
Hb (pH 7)	0.4[c]	1951[a,d]
Mb (pH 3)	<0.01[a]	1966[f]
Hb (pH 3)	<0.01[a]	1966[f]

[a] This work
[b] Defined as 1
[c] [2]
[d] [11]
[e] [12]
[f] [14]
[g] Pd was 12.7 μM
[h] 3.6 mM α-picoline was added

the presence of D-camphor decreased slightly but significantly. Pd was reported to act as an 'effector' on the monooxygenation of D-camphor catalyzed by $P450_{cam}$ [9], though the significance of the present observation is still under investigation. In any case, the D-camphor binding increases the quantum yield of $P450_{cam}$ by ~10-fold.

The binding of organic substances to the camphor binding site does not always increase the quantum yield. In the case of α-picoline, which binds to the camphor binding site [10], the quantum yield did not vary from that in the absence of D-camphor (table 1). Upon inactivation of $P450_{cam}$, the quantum yield decreased to 0.03 (see P420 in table 1) and it was not affected by D-camphor. Such a decrease in the quantum yield after denaturation was observed also with Mb and Hb; the quantum yields for MbCO and HbCO, which were 1 and 0.4, respectively, at pH 7, decreased markedly on the acidification to pH 3 (table 1).

In an attempt to study the mechanism for the alteration of the quantum yield of the heme–CO complex, the infrared spectra of the various forms of

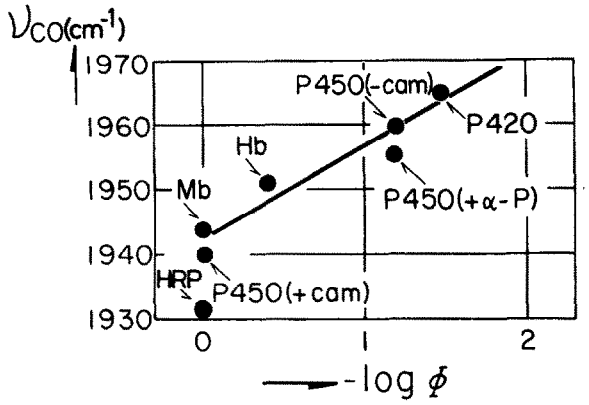

Fig.3. Correlation between the quantum yield and the CO stretching frequency. P450(+cam) and P450(−cam) denote $P450_{cam}$ in the presence and absence of D-camphor, respectively. P450(α-P) denotes $P450_{cam}$ in the presence of α-picoline.

$P450_{cam}$–CO as well as those of CO complexes of Mb and Hb were measured. As is shown in table 1, ν_{CO} found for $P450_{cam}$ were 1940 cm^{-1} in the presence of D-camphor, 1959 cm^{-1} in the absence of D-camphor, 1955 cm^{-1} in the presence of α-picoline and the value for P420 was 1965 cm^{-1}. These values except for that of α-picoline, first measured by us, were in good agreement with those in [13]. By observing ν_{CO} and the quantum yield, we noticed that ν_{CO} shifted toward higher frequency as the quantum yield was reduced. This relationship became more evident when the logarithm of the quantum yield was plotted against the ν_{CO} as in fig.3, in which a linear correlation was obtained.

It was suggested [14] that the shift in ν_{CO} of MbCO and HbCO to a higher value (1966 cm^{-1}) on going from pH 7 to pH 3 was related to a transformation from a bent FeCO bonding in the native protein to more nearly linear bonding in the unfolded protein at pH 3. Furthermore, neutron diffraction and X-ray studies [15,16] revealed that CO ligand for both Mb and Hb in their native forms bound to iron in a bent configuration in consequence of the steric effects of apoproteins. In model compounds, carbonyl–heme–N-base complexes, ν_{CO} were shown to be about 1970 cm^{-1} [11,14], and by X-ray analysis of carbonylheme–pyridine complex, CO was found to be in a linear configuration [17]. Therefore, it is probable that ν_{CO} is related to the configuration of FeCO bonding. Since the quantum yield has been correlated with ν_{CO} (fig.3), it might be associated with the configuration of FeCO bonding.

Given that a shift in ν_{CO} toward lower frequency has been associated with an increase in the π-bonding character of FeCO bond [11,14], the correlation in fig.3 suggests that the increase in π-bonding character enhance the yield for the photodissociation reaction. Therefore, the quantum yield of carbon monoxide hemoproteins might be altered by the bonding character and/or the configuration of the FeCO bonding. An increase in the π-bonding and/or the bent configuration characters is more favourable for the photodissociation.

Acknowledgements

We wish to thank Dr Takaharu Onishi at Tokyo University for the measurements of infrared spectra. We also thank Dr Ryu Makino of our laboratory for the kind supply of the purified horseradish peroxidase. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

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