Interaction of cytochrome b_5 with surfactant vesicles

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Changes in the u.v.–visible absorption spectrum of cytochrome b_5 which occur upon addition of dicetyl phosphate, dimyristoyl phosphatidic acid (DMPA), or mixed DMPA/dimyristoyl phosphatidylcholine vesicles are consistent with haem transfer from the cytochrome to the membrane via an intermediate haemprotein species. The effects of vesicle charge and concentration on the kinetics of haem transfer are discussed.

INTRODUCTION

Cytochrome b_5 is an intrinsic membrane protein composed of two domains that can be separated by proteolytic digestion [1]. The 12000 Da domain is highly polar and contains the haem group. The 3000 Da domain is non-polar and anchors the cytochrome to membranes. Unlike many membrane proteins, cytochrome b_5 is soluble in water in the absence of lipid or detergent [1]. We have recently compared the dithionite reduction of phosphatidylcholine vesicle-bound cytochrome b_5 with that of the free cytochrome [2]. The present study of the interaction of cytochrome b_5 with vesicles was carried out as a prerequisite to further examination of the reaction of aqueous redox partners and the cytochrome bound to charged membranes.

The interaction of cytochrome b_5 with a number of amphiphiles has been studied. At concentrations close to or above their critical micelle concentrations both deoxycholate and dodecyl sulphate bind to the hydrophobic domain of cytochrome b_5 whereas only the dodecyl sulphate associates with the hydrophobic domain of the protein [3] and causes denaturation [4]. The mode of insertion of cytochrome b_5 into membrane vesicles depends upon the method of reconstitution. When the cytochrome is reconstituted into liposomes by the detergent or sonication method the hydrophobic domain inserts in a form that does not transfer between membranes, this has been designated the tight-binding form [5]. When cytochrome b_5 inserts spontaneously into liposomes prepared from certain phospholipids the cytochrome readily exchanges between membranes. This form of the cytochrome has been designated the loose-binding form [5]. The kinetics of the spontaneous binding of cytochrome b_5 to phosphatidylcholine vesicles and the exchange of the loose-binding form of the cytochrome between phosphatidylcholine vesicles have been studied [6,7] by monitoring fluorescence changes. This paper describes changes in the u.v.–visible absorption spectrum of cytochrome b_5 which occur during its interaction with negatively charged vesicles.

MATERIALS AND METHODS

Cytochrome b_5

The protein was isolated from calf liver as the whole protein using minor modifications [8] to a literature procedure [9] and combining fractions from the Sephadex G-75 column with absorbance ratios $A_{280}/A_{413} < 0.39$. Concentrations of solutions of cytochrome b_5 were determined using ϵ_{413} $1.17 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [10]. The protein was dialysed into 0.01 M-Tris/0.005 M-HCl (pH 8.1) buffer before use.

Chemicals

Haem was purchased from Fluka and solutions made up by weight immediately before use. The haem was dissolved in a minimum volume of 0.1 M-NaOH and diluted with 0.01 M-Tris/HCl buffer (pH 8.1). DMPA (approx. 98 %), DMPC (99 + %), and dicetyl phosphate (DCP) were purchased from Sigma.

Vesicles

The required amounts of surfactants were dissolved in chloroform which was removed by extensive flushing with N_2 . Buffer, 0.01 M-Tris/HCl (pH 8.1), was added and sonication carried out using a bath sonifier at 30 °C for DMPC, 60 °C for DMPA and DCP, and 40 °C for mixtures of amphiphiles, until the turbidity of the suspensions reached a minimum. The sonicated vesicles were centrifuged for 30 min at 100000 g and the supernatant used the same day as preparation.

Spectrophotometry

A Hewlett Packard HP 8451A spectrophotometer was used with a 1 cm cell thermostatted at 25 °C. Spectra were recorded after mixing vesicle suspensions with haem or cytochrome b_5 and, unless stated otherwise, corrected for light scattering by subtracting the absorbances observed with vesicles alone at the same concentrations. The spectrophotometer was used to carry out two-component quantitative analyses by fitting the absorbances over the key wavelength range to a

Abbreviations used: haem, iron(III) protoporphyrin IX; CO-haem, carbonmonoxyiron(II) protoporphyrin IX; DMPA, dimyristoyl phosphatidic acid; DMPC, dimyristoyl phosphatidylcholine; DCP, dicetyl phosphate.

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Fig. 1. Changes in the absorbance spectrum of haem after addition of DMPA vesicles

The concentrations of haem and DMPA were $7.7 \mu\text{M}$ and 2 mM respectively. Measurements were made as described in the Materials and methods section at the times indicated.

combination of the spectra of appropriate standards using a least-squares fitting procedure [11].

RESULTS

Haem

Fig. 1 shows the spectral changes which occur on addition of DMPA vesicles to haem. Within the time of mixing the Soret peak intensifies and shifts to 404 nm with a molar absorption coefficient of $8.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

This is followed by a decrease in the absorbance of the Soret peak with no further shift in wavelength and an isosbestic point at 345 nm . The spectral change is exponential and gives a rate constant of $3.8 \times 10^{-4} \text{ s}^{-1}$ and a final molar absorption coefficient of $6.0 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 404 nm . Similar absorbance changes occurred (results not shown) upon addition of DCP vesicles to haem. After the initial rapid increase in the Soret maximum, $\epsilon_{404} 8.6 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$, the peak dropped slowly with a rate constant of $1.3 \times 10^{-4} \text{ s}^{-1}$. Biphasic absorbance changes for the reaction CO-haem with phospholipid vesicles have been interpreted as reflecting coordination of the haem iron by a component of the membrane [12]. Ketterer and co-workers [13] report ϵ_{max} values for phosphatidylcholine- and phosphatidylserine-bound haem of 5.65×10^4 and $7.4 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ respectively. Upon addition of 15 mM -SDS to haem the Soret peak shifted to 402 nm with a molar absorption coefficient of $8.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (results not shown), similar to the value of $9.2 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ reported [13].

Cytochrome b_5

Fig. 2 shows the spectral changes which occur upon addition of DMPA vesicles to cytochrome b_5 . The Soret peak drops and shifts from 414 nm to 408 nm and a shoulder develops around 370 nm . The decay of the isosbestic point indicates that there are at least two stages in the reaction. Addition of solid sodium deoxycholate reverses the spectral changes caused by DMPA vesicles as shown in Fig. 3. The background absorbance decreases owing to the formation of DMPA-deoxycholate comices; the relative intensity of the Soret band increases and the peak shifts back to 412 nm (changes in the background absorbance do not modulate λ_{max} values). The interaction of SDS with cytochrome b_5 , illustrated in Fig. 4 is also reversible. Upon addition of 15 mM -SDS the Soret peak of the cytochrome shifts to 404 nm and a shoulder develops around 370 nm resulting in a spectrum

Fig. 2. Changes in the absorbance spectrum of cytochrome b_5 after addition of DMPA vesicles

The concentrations of cytochrome and phosphatidic acid were $2.68 \mu\text{M}$ and 2 mM respectively. Measurements were made at the times indicated.

Fig. 3. Effect of deoxycholate on the absorbance spectrum of cytochrome b_5 14 h after mixing with DMPA vesicles

Spectra of 2.68 μM -cytochrome b_5 14 h after mixing with 2 mM-DMPA vesicles (—), and after addition of sodium deoxycholate (-----). Vesicle background absorbances have not been subtracted.

Fig. 4. Changes in the absorbance spectrum of cytochrome b_5 after addition of SDS and subsequent dilution

Spectra of 7.5 μM -cytochrome b_5 divided by 10 (.....), 7.5 μM -cytochrome b_5 in 15 mM-SDS divided by 10 (-----) and previous sample after 10-fold dilution (—).

very similar to that of SDS-bound haem. Dilution of the mixture to give an SDS concentration of 1.5 mM, below the critical micelle concentration [14], causes regeneration of the original spectrum of cytochrome b_5 . Addition of 50 and 20 mol% DMPA/DMPC vesicles and DCP vesicles to cytochrome b_5 caused similar spectral changes (not shown) to those observed with DMPA. No spectral changes occurred after addition of 10 mol% DMPA/

Fig. 5. Spectrum of cytochrome b_5 after addition of DCP vesicles

Spectrum of 2.68 μM -cytochrome b_5 30 min after mixing with 1 mM-DCP vesicles (—). Sum of the spectra of 1.01 μM -cytochrome b_5 and 1.63 μM -DCP-bound haem (-----).

DMPC or DMPC vesicles and the vesicle background-subtracted spectra were identical with that of free cytochrome b_5 .

After the initial binding, the absorbance spectrum of haem in DCP vesicles changes very slowly, as described in the previous section. We therefore used the initial spectrum of DCP-bound haem to carry out a two-component analysis of the spectrum obtained 30 min after addition of DCP vesicles to 2.68 μM -cytochrome b_5 . Cytochrome b_5 and DCP-bound haem concentrations were calculated using absorbances between 400 and 420 nm. The theoretical spectrum corresponding to the calculated concentrations is compared to the observed spectrum in Fig. 5. The similarity of the spectra suggests that cytochrome b_5 is partially converted to membrane-bound haem and, or alternatively, a species with similar spectral characteristics to membrane-bound haem.

Biphasic absorbance changes occurred after mixing cytochrome b_5 with vesicles. Absorbances at 414 nm were fitted to eqn. (1) using non-linear least squares; the results are summarized in Table 1.

$$A_{414} = A_{\infty} + \Delta A \cdot e^{-kt} + \Delta A' \cdot e^{-k't} \quad (1)$$

The kinetic parameters are not markedly dependent on the composition of mixed DMPA/DMPC vesicles in the range 100% to 20% DMPA. No absorbance changes occurred, however, with 10% DMPA vesicles or with DMPC vesicles. The rate constant, k , of the initial rapid phase is greater for DCP than DMPA vesicles. It is notable that although the concentration of DCP has little effect on the rate constants k and k' the magnitude of the initial absorbance change, ΔA , is dependent upon vesicle concentration. The slow phase of the interaction of cytochrome b_5 with DCP vesicles has a rate constant, k' , much greater than $1.3 \times 10^{-4} \text{ s}^{-1}$, which is the rate constant for the process occurring subsequent to haem binding to DCP. So the biphasic time course observed with cytochrome b_5 and DCP is not due to the change which

Table 1. Kinetic parameters for the interaction of cytochrome b_5 with vesicles

The concentration of cytochrome b_5 was $2.68 \mu\text{M}$ except where stated otherwise. The experimental conditions are described in the Materials and methods section. The kinetic parameters are defined in eqn. (1). * $2.64 \mu\text{M}$ -cytochrome b_5 , † $2.79 \mu\text{M}$ -cytochrome b_5 .

Vesicle composition	Vesicle concentration (mM)	ΔA	$\Delta A'$	A_∞	$10^3 \times k$ (s ⁻¹)	$10^3 \times k'$ (s ⁻¹)
DMPA	2	0.050	0.046	0.207	7.0	0.8
50 % DMPA/DMPC*	2	0.043	0.051	0.197	7.0	0.6
20 % DMPA/DMPC†	2	0.043	0.060	0.193	9.5	0.5
10 % DMPA/DMPC	2	—	—	—	0	0
DMPC	2	—	—	—	0	0
DCP	2	0.088	0.032	0.210	27.0	1.3
DCP	1	0.046	0.028	0.230	30.0	1.8

occurs to the haem in the membrane. The rate constants for the slow phase of the interaction of cytochrome b_5 with DMPA and that of haem with DMPA are 8.2×10^{-4} and $3.8 \times 10^{-1} \text{ s}^{-1}$ respectively. Biphasic processes for which each phase has a similar rate constant appear monophasic with an apparent rate constant between the two actual values. Hence it is possible that the slow phase of the interaction of cytochrome b_5 with DMPA includes a contribution from the interaction of haem with the vesicles subsequent to its transfer from the protein. Therefore no further quantitative treatment of the data for the DMPA vesicles has been attempted.

DISCUSSION

The spectral changes which occur upon addition of DMPA, 50 % DMPA/DMPC, 20 % DMPA/DMPC and DCP vesicles to cytochrome b_5 are consistent with haem transfer from the cytochrome to the negatively charged vesicles. DMPC and 10 % DMPA/DMPC do not remove haem from the cytochrome. It may seem surprising that the more negatively charged vesicles remove haem from the cytochrome since they have a slightly lower affinity for haem than the neutral vesicles due to unfavourable electrostatic interactions with the negative haem moiety [13]. The charged membrane must facilitate the dissociation of the haem from the cytochrome by perturbing the conformation of the protein.

Addition of sodium deoxycholate reverses the spectral change which occurs upon the interaction of negatively charged vesicles with cytochrome b_5 . Disruption of the protein-lipid interaction by deoxycholate could enable the apoprotein to regain a conformation with a higher affinity for haem than that of the deoxycholate micelles. This would allow the haem to reassociate with the protein. The fact that deoxycholate interacts only with the hydrophobic domain of cytochrome b_5 [3] and does not cause disassociation of the haem from the protein shows that haem has a higher affinity for the protein than for deoxycholate. Cytochrome b_5 does not interact with SDS below its critical micelle concentration, but above its critical micelle concentration, SDS binds to the hydrophobic and haem-containing domains of the protein and causes denaturation [3,4]. We have shown that the interaction of cytochrome b_5 and SDS is reversible and analogous to the interaction of the protein with negatively charged membranes.

The biphasic kinetics for the interaction of cytochrome b_5 with DCP vesicles can be explained according to eqns. (2) and (3).

Cytochrome b_5 , its intermediate form with a lowered affinity for haem, and the haem moiety are represented by b , I and H respectively. The further change which occurs to the haem in the membrane is slow compared with the processes represented in eqns. (2) and (3) and can be neglected. Theoretical time courses corresponding to eqns. (2) and (3) were generated by numerical integration. Absorbance changes were treated using a non-linear least-squares fitting procedure to eqn. (4).

$$A_{414} = \epsilon_b[b] + \epsilon_I[I] + \epsilon_H[H] \quad (4)$$

ϵ_b was taken as $1.17 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [10] and ϵ_H as $6.44 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (calculated from the initial absorbance of DCP-bound haem since, because of the high affinity of haem for membranes [13], most of the haem released from cytochrome b_5 is membrane-bound). ϵ_I is not known and so a range of values was used in the calculation: $4.8 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ from the spectrum of the high-spin form of cytochrome b_5 seen at high temperature [15]; $2.43 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ from the high-spin form seen at high pH [16]; and $6.44 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ the absorption coefficient of DCP-bound haem. The latter value was used because the spectrum of the intermediate formed during the reaction of globin and CO-haem to form carbonmonoxyhaemoglobin is reported to be analogous to that of CO-haem incorporated into membranes [17]. Table 2 shows that the values of k_{+1} and k_{+2} approximately double over the range of ϵ_I values used in the calculation and the values of k_{-1} and k_{-2} are less dependent on ϵ_I . The effects of DCP concentration on the rate constants are similar for each of the ϵ_I values used and we conclude that the ranges of rate constants obtained are reasonable approximations to the true values provided that eqns. (2) and (3) reflect the actual mechanism.

The biphasic spectral changes observed upon addition of negatively charged vesicles to cytochrome b_5 and the reversal of these changes by deoxycholate are consistent

Table 2. Rate constants for the reaction of cytochrome b_5 with DCP vesicles

Details are given in the text. The concentration of cytochrome b_5 was $2.68 \mu\text{M}$. The experimental conditions are described in the Materials and methods section. The rate constants are defined in eqns. (2) and (3).

[DCP] (mM)	$10^{-4} \times \epsilon_1$ ($\text{M}^{-1} \cdot \text{cm}^{-1}$)	$10^3 \times k_{+1}$ (s^{-1})	$10^3 \times k_{-1}$ (s^{-1})	$10^3 \times k_{+2}$ (s^{-1})	$10^{-2} \times k_{-2}$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
2	2.43	6.8	15.0	1.40	7.5
2	4.80	9.2	12.7	1.10	6.8
2	6.44	12.0	10.1	0.85	6.4
1	2.43	7.4	23	1.7	16
1	4.80	9.9	21	1.3	15
1	6.44	13.0	18	1.0	14

with an alternative mechanism in which the haem remains associated with the protein in a number of conformational variants. We feel that this mechanism is less likely than that represented by eqns. (2) and (3) for the following reasons. Firstly, it is well known that the formation constants of mono(nitrogen ligand) Fe(III) porphyrin complexes are very much less than those of the bis(nitrogen ligand) complexes, e.g. see [18], concerning imidazole-haem complexes in ethanol/water mixtures. Hence, once the first imidazole-haem bond of cytochrome b_5 is broken due to the influence of the negatively charged membrane, the second imidazole-haem bond can break much more easily and since the haem has a high affinity for the membrane it will partition into that phase. Secondly, the thermal denaturation of cytochrome b_5 in aqueous solution results in a number of conformational variants distinguishable by Raman spectroscopy [15]. When the protein was kept at 62°C for 1.5 h evidence for a dissociated free haem was obtained [15].

The pseudophase model of Berezin and co-workers [19] is used to treat the effect of DCP concentration on the kinetics. Cytochrome b_5 , the intermediate, haem, and apocytochrome b_5 are considered to be partitioned between a vesicle phase and the bulk aqueous phase. The respective Soret spectra of cytochrome b_5 and the intermediate in the aqueous and vesicle phases are assumed to be identical and hence each of the reactions shown in eqns. (2) and (3) is considered to be the sum of reactions occurring in the aqueous and vesicle phases. For the unimolecular reactions involving b or I it can be shown that:

$$k_i = \frac{k_i^v \cdot P_x \cdot \bar{V} \cdot [\text{DCP}] + k_i^w}{1 + P_x \cdot \bar{V} \cdot [\text{DCP}]} \quad (5)$$

and for the bimolecular reaction of apocytochrome b_5 and haem:

$$k_{-2} = \frac{k_{-2}^v \cdot P_H \cdot P_{\text{apob}} \cdot \bar{V} \cdot [\text{DCP}] + k_{-2}^w}{(1 + P_H \cdot \bar{V} \cdot [\text{DCP}]) \cdot (1 + P_{\text{apob}} \cdot \bar{V} \cdot [\text{DCP}])} \quad (6)$$

where k_i represents k_{+1} , k_{-1} or k_{+2} and P_x represents the partition coefficient of x (x is b or I) between the vesicle and aqueous phases. P_H is the partition coefficient of haem. The superscripts v and w indicate rate constants in the vesicle and aqueous phases respectively. $\bar{V}$ is the molar volume of the vesicle phase of DCP. The vesicle phase may be a region, at the interface of the membrane

and the aqueous solution, which includes the vesicle head-group region as suggested [13] for membrane-bound haem. It is possible to estimate $\bar{V}$ from the dimensions [20] of DCP vesicles, assuming that the head-group region is about 0.5 nm thick, a value of $\bar{V} 0.05 \text{ M}^{-1}$ is obtained. Since the affinity of haem for the vesicle phase is much greater than for the aqueous phase, i.e. $P_H \cdot \bar{V} \cdot [\text{DCP}] \ll 1$, eqn. (6) reduces to:

$$k_{-2} = \frac{k_{-2}^v \cdot P_H \cdot P_{\text{apob}} \cdot \bar{V} \cdot [\text{DCP}] + k_{-2}^w}{P_H \cdot \bar{V} \cdot [\text{DCP}] \cdot (1 + P_{\text{apob}} \cdot \bar{V} \cdot [\text{DCP}])} \quad (7)$$

A comparison of the effect of DCP concentration on the rate constants (Table 2) with those predicted from eqns. (5) and (7) suggests the following. Cytochrome b_5 , the intermediate, and apocytochrome b_5 have a high affinity for the vesicle phase, i.e. $P_b, P_i, P_{\text{apob}} \gg 1/\bar{V} \cdot [\text{DCP}]$. The formation of the intermediate from cytochrome b_5 , loss of haem from the intermediate, and association of haem and apocytochrome b_5 occur predominantly in the vesicle phase, i.e. $k_{+1}^v \cdot P_b/k_{-1}^w, k_{+2}^v \cdot P_i/k_{-2}^w, k_{-2}^v \cdot P_H \cdot P_{\text{apob}}/k_{-2}^w \gg 1/\bar{V} \cdot [\text{DCP}]$. The reversion of the intermediate to cytochrome b_5 , however, occurs mainly in the aqueous phase, $k_{-1}^v \cdot P_i/k_{-1}^w \ll 1/\bar{V} \cdot [\text{DCP}]$. This indicates that the affinity of cytochrome b_5 for the vesicle phase is less than that of the intermediate.

The transfer of haem between phosphatidylcholine vesicles [12] and uptake of haem from vesicles by aqueous haem-binding proteins [12,21] is limited by the rate of dissociation of the haem from the membrane. Our results were not consistent with a mechanism which included rate-limiting dissociation of haem from the membrane followed by association of the haem and apocytochrome b_5 in the aqueous phase. This observation may have some bearing on the role, or lack of one, of haem-binding proteins in the intracellular transport of haem [22,23]. It has been suggested [12] that cytoplasmic haem-binding proteins, such as ligandin and glutathione-S-transferase, have little to do with catalysing the migration of haem from the mitochondrial inner membrane to the endoplasmic reticulum but may be required to prevent dispersal of haem within the cell and extracellular loss. The kinetic results reported here indicate that the transfer of haem from the membrane to cytochrome b_5 takes place in the membrane phase and does not involve haem entering the aqueous phase.

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