Kinetics and mechanism of electron transfer from dithionite to microsomal cytochrome b_5 and to forms of the protein associated with charged and neutral vesicles

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The kinetics of the dithionite reduction of calf liver microsomal cytochrome b_5 , both free in solution and bound to dimyristoyl phosphatidylcholine vesicles, are consistent with electron transfer between SO_2^- and the exposed haem edge of the protein. The vesicle membrane does not hinder the approach of SO_2^- to the site of electron transfer on the protein. In 0.01 M-Tris/HCl buffer, pH 8.1, k_{et} (25 °C), $\Delta H_{\text{et}}^\ddagger$ and $\Delta S_{\text{et}}^\ddagger$ are estimated to be $1.44 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, $7.8 \text{ kJ} \cdot \text{mol}^{-1}$ and $-92.3 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ respectively. The cytochrome exhibits an acid dissociation, $\text{p}K_{\text{a}} 9.3 \pm 0.3$, and the rate of electron transfer from dithionite to the high-pH form is about one-third of that to the neutral-pH form. The effect of ionic strength on the kinetics is consistent with a reaction between like-charged species and is discussed in terms of a number of theoretical models. In systems comprising cytochrome b_5 and negatively charged vesicles, the effect of increasing the charge density of mixed dimyristoyl phosphatidylcholine/dicetyl phosphate vesicles and of increasing the concentration of dicetyl phosphate vesicles is to lower the rate of electron transfer from dithionite to the haem moiety of the cytochrome. With vesicles of high charge density, however, the kinetics are complicated by vesicle-induced conformation changes of the cytochrome.

INTRODUCTION

Microsomal cytochrome b_5 is an amphipathic protein consisting of a negatively charged redox-active haem-containing domain separated by a peptide linkage of about seven residues from a smaller hydrophobic domain that attaches the protein to the membrane. The structure and properties of cytochrome b_5 have recently been reviewed [1]. The study of the reactions of redox proteins and small redox partners is an important approach to the characterization of electron transfers in biological systems [2], and a number of such studies concerning the haem-containing segment of cytochrome b_5 released by trypsin digestion (the haem peptide) have been reported [3–7]. It has further been shown that the rates of dithionite reduction of the haem peptide and the whole cytochrome in solution and bound to egg-phosphatidylcholine vesicles are very similar [8,9].

In the present paper we report studies including the effect of pH, ionic strength, temperature and binding to neutral and charged membranes on the kinetics of the dithionite reduction of the haem moiety of cytochrome b_5 . A preliminary account of some of this work has appeared [9].

EXPERIMENTAL

Materials

The isolation of cytochrome b_5 from calf liver has been described previously [10]. The purified protein was diluted to about $12 \mu\text{M}$ and stored at -20°C . It was dialysed into the appropriate buffer before use.

Vesicles were prepared as described previously [10] and, if required, diluted with buffer. Binding of cytochrome b_5 to the vesicles was effected by incubation at 28°C under N_2 for 2–18 h. Gel filtration on Bio-Gel A-1.5m indicated that binding was essentially complete at the higher molar ratios of surfactant to cytochrome.

$\text{Na}_2\text{S}_2\text{O}_4$ was either a gift from Dr. R. N. F. Thorneley, A.F.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K., or purchased from BDH Chemicals. It was assayed in the stopped-flow system under anaerobic conditions by spectrophotometric titration against a slight excess of $\text{K}_3\text{Fe}(\text{CN})_6$ by using $\epsilon_{418} 1012 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [11]. Chemicals were of the best available grade.

Kinetics

A Hi-Tech SF-2A stopped-flow spectrophotometer was modified for O_2 -free work by flushing O_2 -free N_2 through a phosphate-buffered dithionite solution in the thermostat bath, which was sealed from the atmosphere. Teflon components of the flow system that were in contact with air were covered with pure aluminium adhesive tape.

Solutions were deoxygenated by flushing with water-saturated Ar or O_2 -free N_2 scrubbed with Fieser's solution [12]. Deoxygenated solutions were transferred under gas pressure with the use of double-ended stainless-steel needles and serum-capped vessels. $\text{Na}_2\text{S}_2\text{O}_4$ added to a dry vessel was dissolved in deoxygenated buffer. Before kinetic runs the flow system was flushed with dithionite solution in order to remove any O_2 adsorbed on internal surfaces.

Abbreviations used: haem, iron(III) protoporphyrin IX; DMPC, dimyristoyl phosphatidylcholine; DCP, dicetyl phosphate; SA, stearylamine.

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Reactions were carried out with 1–2 μM -cytochrome b_5 , unless stated otherwise. Dithionite was in at least 50-fold excess. Transmittance changes at 424 nm, corresponding to the reduction of cytochrome b_5 , were digitized and stored by using a 16-bit-resolution CIL Microsystems PCI 6380 interface unit that was connected to a Commodore 4032 microcomputer. A simple program displayed plots of $\ln(A_\infty - A)$ versus time and calculated first-order rate constants, $k_{\text{obs.}}$, from the slopes of the plots. Rate constants were determined from the average values for five consecutive runs.

RESULTS AND DISCUSSION

Rate law and effect of uncharged vesicles

The reduction of 1.25 μM -cytochrome b_5 free in solution and bound to 2 mM-dimyristoyl phosphatidylcholine (DMPC) vesicles by excess dithionite was studied over a wide range of dithionite concentrations at 25 °C in 0.01 M-Tris/0.005 M-HCl buffer, pH 8.1 and I 0.005, and in 0.01 M-Tris/0.005 M-HCl/0.05 M-NaCl buffer, pH 8.1 and I 0.055. Plots of $\ln(A_\infty - A)$ versus time show that the reactions conform to first-order kinetics with respect to cytochrome b_5 concentration for more than 3 half-lives. Plots of the pseudo-first-order rate constant, $k_{\text{obs.}}$, against the square root of the dithionite concentration are shown in Fig. 1 of reference [9] to be linear and to extrapolate through the origin. Thus the reaction exhibits the rate law given in eqn. (1):

$$-\frac{d[\text{cyt. } b_5(\text{III})]}{dt} = k[\text{cyt. } b_5(\text{III})][\text{S}_2\text{O}_4^{2-}]^{\frac{1}{2}} \quad (1)$$

where:

$$k_{\text{obs.}} = k[\text{S}_2\text{O}_4^{2-}]^{\frac{1}{2}} \quad (2)$$

The rate law is consistent with the mechanism shown in eqns. (3) and (4):

where:

$$k = k_{\text{et}} \cdot K^{\frac{1}{2}} \quad (5)$$

Similar mechanisms have been proposed for the dithionite reduction of other electron-transfer proteins (see, e.g., [13–15]).

Rate constants, k , expressed as (mean $\pm$ S.D.) $\text{M}^{-\frac{1}{2}} \cdot \text{s}^{-1}$ for membrane-bound and free cytochrome b_5 respectively are 112 ± 10 and 130 ± 8 in medium of I 0.055 and 62 ± 8 and 60 ± 3 in medium of I 0.005. The similarity of the rate constants for the reduction of free and formally neutral membrane-bound cytochrome b_5 described here and elsewhere [8] shows that the approach of SO_2^- to the site of electron transfer of the membrane-bound protein is unhindered by the membrane. This result is consistent with the model of membrane-bound cytochrome b_5 proposed by Kleinfeld & Lukacovic [16], in which the haem-containing domain abuts the membrane surface with the partially exposed haem edge facing away from the membrane. A further discussion of this point is included in the preliminary account of this work [9].

A similar value of k to that of cytochrome b_5 is observed for the dithionite reduction of metmyoglobin [13,15]. From eqn. (5), by using a value of K 1.86×10^{-9} M calculated from the ionic-strength-dependence [14], a value of k_{et} $1.44 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ is calculated for the

Table 1. Effect of cytochrome b_5 concentration on the pseudo-first-order rate constant

Runs were carried out in 0.01 M-Tris/0.005 M-HCl buffer, NaCl buffer, pH 8.1, at 25 °C. The dithionite concentration was 0.28 mM.

[Cytochrome b_5] (μM)	$k_{\text{obs.}}$ (s^{-1})
0.81	2.28
1.38	2.25
2.14	2.23
2.69	2.19
4.41	1.67

reaction of SO_2^- with DMPC-vesicle-bound cytochrome b_5 at I 0.005.

Effect of cytochrome b_5 concentration

Under certain conditions the hydrophobic domain of cytochrome b_5 causes the protein to aggregate to form micelle-like octameric structures [17]. The concentration-dependence study shown in Table 1 was carried out to test whether octamer formation, if it occurs under the present conditions, influences the kinetics of the dithionite reduction of the protein. Table 1 shows that the protein concentration does not affect $k_{\text{obs.}}$, except at the highest concentration, which suggests that the protein is monomeric and below its 'critical micelle concentration' under the conditions generally employed in the present work. This is consistent with the report [8] that the rate of dithionite reduction of cytochrome b_5 is very similar to that of the haem peptide, which does not aggregate.

Effect of Na^+ concentration

On the basis of X-ray-crystallographic studies of oxidized and reduced cytochrome b_5 , Argos & Mathews [18] have proposed a mechanism for the reduction of the protein that involves a cation, such as Na^+ , binding to the negative charge on an exposed haem propionate group upon reduction of the iron centre. Table 2 shows that at constant ionic strength the NaCl concentration does not affect $k_{\text{obs.}}$ for the dithionite reduction of cytochrome b_5 , and hence it is unlikely that Na^+ binding occurs at the exposed haem edge of the protein upon reduction. Experiments utilizing dimethyl ester haem-substituted cytochrome b_5 [5] also throw doubt on the model proposed by Argos & Mathews [18].

Table 2. Effect of Na^+ concentration on the pseudo-first-order rate constant

Runs were carried out in Tris/HCl/NaCl buffer, pH 8.1 and I 0.105 at 25 °C. The dithionite concentration was 0.28 mM.

[NaCl] (M)	$k_{\text{obs.}}$ (s^{-1})
0	2.86
0.025	2.56
0.050	2.74
0.075	2.75
0.100	2.80

Table 3. Rate constants for the dithionite reduction of cytochrome b_5 measured as a function of ionic strength

Runs were carried out at 25 °C in 0.01 M-Tris/0.005 M-HCl buffer, pH 8.1, with the ionic strength adjusted with NaCl. N is the number of different dithionite concentrations used. Quoted rate constants are the averages for N determinations.

I	k ($\text{M}^{-\frac{1}{2}} \cdot \text{s}^{-1}$)	N
0.005	59.6	5
0.015	89.3	3
0.025	93.1	3
0.045	111	4
0.050	102	1
0.055	103	1
0.065	124	1
0.070	118	1
0.075	117	1
0.119	178	1
0.133	159	1
0.160	160	1
0.209	203	3
0.311	241	1
0.416	273	1
0.536	319	1
0.633	321	1
0.761	308	1
1.016	327	1

The results in Table 2 show that the kinetic-ionic-strength study reported below is not complicated by specific Na^+ effects.

Effect of ionic strength

The effect of ionic strength on the overall three-halves-order rate constant for the dithionite reduction of cytochrome b_5 is shown in Table 3. The data are fitted to the Brönsted-Debye-Huckel (BDH) and Marcus Ionic Strength (MIS) equations derived by Wherland & Gray [19] and modified to take into account the ionic-strength-dependence of the dissociation constant, K , of dithionite [14]. When values for the respective radii of SO_2^- and cytochrome b_5 of 0.253 [14] and 1.59 nm, the latter calculated from the molecular mass of the haem-containing domain of the protein [3], are substituted into the BDH and MIS equations, the values of the charge on the protein shown in lines 1 and 2 of Table 4 are

obtained. These are in good agreement with the values of -9 calculated [6] from the amino acid sequence of the haem-containing domain. If, however, the radius of the protein, in addition to its charge and $K_0^{\frac{1}{2}} \cdot k_0$ or $K_0^{\frac{1}{2}} \cdot k_\infty$ (K_0 is K at zero ionic strength and k_0 and k_∞ are respectively k_{et} at zero and infinite ionic strengths), is made an adjustable parameter then better fits, as shown by the lower sums of squares of deviations, to the equations are obtained (Table 4, lines 3 and 4). These solutions to the equations, however, are inconsistent with the model used to derive the equations [19], which treats the protein as a uniformly charged sphere, since such a sphere should have charge and radius corresponding to those of the protein, i.e. approx. -9 and 1.59 nm respectively.

An equation derived by Watkins and co-workers [20] and modified to take into account the ionic-strength-dependence of the dissociation constant, K , of dithionite [14] can be used to fit the data shown in Table 3 without leading to the self-contradiction obtained for the best-fit solutions of the BDH and MIS equations. The Watkins equation is derived from a model that treats the interaction domain of the protein and its redox partner as parallel discs, radius ρ , at a distance R_{12} with an effective interaction-site charge of the protein Z_1 and effective dielectric constant within the interaction domain D_e . When values of R_{12} 0.35 nm and D_e 78, which is the bulk dielectric constant of water, are substituted into the equation the best-fit values of ρ and Z_1 given in line 5 of Table 4 are obtained. There is [1] no region of the protein with such a high charge density as is implied by the values of ρ and Z_1 . If, however, as appears reasonable [20], a smaller value of D_e is used, then, without affecting the best-fit values of $K_0^{\frac{1}{2}} \cdot k_\infty$, a proportionately smaller protein interaction-domain charge is obtained, which is in keeping with the topology of the cytochrome.

The MIS and Watkins equations provide equally good fits to the kinetic-ionic-strength data, as shown by the sums of squares of deviations in lines 4 and 5 of Table 4. The corresponding values of $K_0^{\frac{1}{2}} \cdot k_\infty$ are in good agreement, and by using K_0 2.14×10^{-9} M estimated from the data of [14] a value of k_∞ $1.2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ is obtained. This represents the value of k_{et} unperturbed by the electrostatic work term involved in bringing the SO_2^- ion to the site of electron transfer on the protein.

Effect of pH

Studies of the reaction of cytochrome b_5 with CoEDTA^- [6] and FeEDTA^{2-} [3] in the pH region 5.5–8

Table 4. Parameters calculated from kinetic-ionic-strength data

For the Watkins equation protein radius and charge refer to radius, ρ , and charge, Z_1 , of the protein interaction domain (see the text). Abbreviation: SSD, sum of squares of deviations.

Data treatment	Protein radius (nm)	Protein charge	$K_0^{\frac{1}{2}} \cdot k_0$ ($\text{M}^{-\frac{1}{2}} \cdot \text{s}^{-1}$)	$K_0^{\frac{1}{2}} \cdot k_\infty$ ($\text{M}^{-\frac{1}{2}} \cdot \text{s}^{-1}$)	SSD ($\text{M}^{-1} \cdot \text{s}^{-2}$)
BDH. Protein radius fixed	1.59	-10.7	11.5	—	10477
MIS. Protein radius fixed	1.59	-9.4	—	448	14061
BDH. Protein radius adjustable	0.70	-3.9	36.6	—	4754
MIS. Protein radius adjustable	0.65	-3.4	—	581	4198
Watkins equation	0.45	-9.3	—	564	4179

Fig. 1. Effect of pH on the pseudo-first-order rate constant

Runs were carried out at 25 °C in the following buffers: ○, Tris/HCl buffer, 1.005; ●, Tris/HCl/NaCl buffer, 1.055; □, carbonate/bicarbonate buffer, 1.005. The dithionite concentrations were 0.60 mM for the runs carried out at the higher ionic strength (●) and 0.67 mM at the lower ionic strength. The curve represents the best fit to eqn. (6).

show increases in rate with decreasing pH, which have been attributed to ionizable groups on the protein with pK_a 5.8 and 5.2 respectively. Similarly, we observed an increase in the rate of the dithionite reduction of cytochrome b_5 below pH 7. The dithionite, however, is not stable under these conditions, and reliable rate constants were not obtained (results not shown).

Fig. 1 shows the pH-dependence of the rate of dithionite reduction of cytochrome b_5 in the pH range 7–10. The region above pH 8 is of particular interest, since no other kinetic studies have been carried out in this region. The dependence of k_{obs} on pH is fitted to eqn. (6):

$$k_{\text{obs.}} = \frac{k_1[\text{H}^+] + k_2 K_a}{[\text{H}^+] + K_a} \quad (6)$$

where k_1 and k_2 are the rate constants for the neutral-pH and high-pH forms of the protein and K_a is the acid dissociation constant of the neutral form, to give k_1 $1.42 \pm 0.08 \text{ s}^{-1}$, k_2 $0.45 \pm 0.23 \text{ s}^{-1}$ and pK_a 9.3 ± 0.3 . Studies of paramagnetic susceptibility, e.s.r. and u.v.-visible spectrophotometry of cytochrome b_5 as a function of pH indicate no changes in the environment of the haem in the pH region 8–10 [21,22]. Therefore the observed pK_a is attributed to deprotonation of one of the positively charged residues on the surface of the protein. This leads to an increased repulsion between the incoming SO_2^- and the negatively charged protein during electron transfer, which results in a decrease in the rate constant.

Effect of temperature

Fig. 2 shows the temperature-dependence of the overall three-halves-order rate constant for the dithionite reduction of cytochrome b_5 . In order to obtain the enthalpy, $\Delta H_{\text{et}}^\ddagger$, and entropy, $\Delta S_{\text{et}}^\ddagger$, of activation for electron transfer from SO_2^- to cytochrome b_5 , the

Fig. 2. Eyring plot for the reduction of DMPC-bound cytochrome b_5 by dithionite

Runs were carried out in 0.01 M-Tris/0.005 M-HCl buffer, pH approx. 8, at 25 °C. The concentrations of DMPC and dithionite were 2 mM and 0.74 mM respectively.

temperature-dependence of the dissociation constant, K , of dithionite must be taken into account. By using values of the enthalpy and entropy of dithionite dissociation $83.3 \text{ kJ} \cdot \text{mol}^{-1}$ and $110 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ calculated from the temperature-dependence of K [14] values of $\Delta H_{\text{et}}^\ddagger$ $7.8 \text{ kJ} \cdot \text{mol}^{-1}$ and $\Delta S_{\text{et}}^\ddagger$ $-92.3 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ are obtained. These activation parameters are similar to those for the reaction of cytochrome b_5 with FeEDTA^{2-} [3], CoEDTA^- [6] and ferric nitrilotriacetate [7] and consistent with electron transfer at the exposed haem edge but they are significantly different from those for the reaction of the cytochrome with $\text{Cu}(\text{nitrilotriacetate})^-$ [7], for which binding of the metal co-ordination complex to a site on the side of the protein occurs [7], and it is postulated [7] that long-range electron transfer occurs subsequently to binding.

Effect of charged vesicles

It is reported [23] that in reconstituted systems the vesicle charge controls the rate of electron transfer from NADPH-cytochrome $P-450$ to cytochromes b_5 , $P-450_{\text{LM}2}$ and $P-450_{\text{LM}4}$ but not to cytochrome $P-450_{\text{LM}3}$. The following is an attempt to determine the effect of vesicle charge on the dithionite reduction of cytochrome b_5 , which we initially thought to be a relatively simple system involving, for negatively charged vesicles, electron transfer between SO_2^- , predominantly in the aqueous phase, and cytochrome b_5 , partitioned between the aqueous and vesicle phases.

Fig. 3 shows the effect of increasing the proportion of dicetyl phosphate (DCP), which is negatively charged, on the rate constant and the absorbance change, ΔA , occurring during the reduction of cytochrome b_5 bound to mixed DMPC/DCP vesicles. In the region 0–20 mol % DCP the rate constant decreases as the proportion of DCP is increased. This is consistent with the negatively charged dithionite being progressively repelled from the surface region of the vesicles, which contains the cytochrome, as the charge on the vesicles is increased. This leads to a decreased effective concentration of the reductant in contact with the cytochrome and hence a lowering of the rate of electron transfer. The decrease in rate constant is, however, much less than that reported for similar studies of the ascorbate/cytochrome c system

Fig. 3. Effect of vesicle charge on the dithionite reduction of cytochrome b_5 bound to mixed DMPC/DCP vesicles

Runs were carried out in 0.01 M-Tris/0.005 M-HCl buffer, pH 8.1, at 25 °C. The dithionite concentration was 0.26 mM at 5 mol %, 10 mol % and 20 mol % DCP and 0.72 mM for the other points. The total surfactant concentration was 1 mM and the concentration of cytochrome b_5 was 1.25 μ M. $\circ$, $k_{\text{obs}}/[S_2O_4^{2-}]^{1/2}$; $\bullet$, ΔA .

Fig. 4. Effect of DPC concentration on the dithionite reduction of cytochrome b_5

Runs were carried out in 0.01 M-Tris/0.005 M-HCl buffer, pH 8.1, at 25 °C. The dithionite and cytochrome b_5 concentrations were 0.48 mM and 1.25 μ M respectively. $\circ$, $1/(k_w - k_{\text{obs}})$; $\bullet$, ΔA .

[24], in which cytochrome c is tightly bound electrostatically to the membrane surface. The haem-containing domain of cytochrome b_5 may not be as tightly constrained at the vesicle surface as is that of cytochrome c . The interdomain linkage of about seven amino acid residues, which joins the haem-containing domain to the hydrophobic membrane-binding domain, may be flexible [1] and allow the negatively charged haem-containing domain, as the membrane charge is increased, to move away from the membrane surface into a region of higher reductant concentration, thus modulating the effect of surface charge on the rate of reduction of the protein.

In the range 20–25 mol % DCP ΔA is sharply decreased (Fig. 3). We have recently reported [10] that in relatively highly charged vesicle systems cytochrome b_5 undergoes a sharp conformational transition over a small range of increasing negative charge. This conformational change results in the haem equilibrating between protein-bound and membrane-bound states. The changes in rate constant observed above 25 mol % DCP reflect reduction of haem species other than, or in addition to, cytochrome b_5 in its native conformation, and definitive interpretation of the data cannot be made at present.

Fig. 4 shows the effect of DCP concentration upon the observed rate constant and ΔA . Consideration of the pseudo-phase model proposed by Berezin and co-workers [25] for a simple model in which the native form of the protein is partitioned between the bulk aqueous phase and vesicle phase to an extent that depends upon the concentration of the vesicles and in which the dithionite is predominantly partitioned into the bulk aqueous phase as a result of electrostatic repulsion from the vesicles leads to the prediction that a plot of $1/(k_w - k_{\text{obs}})$, where k_w is the rate constant in the absence of vesicles, versus $1/[DCP]$ should be linear. Fig. 4 shows that this prediction does not hold, and, clearly, the simple model above does not describe the system adequately. The gradual decrease of ΔA as the concentration of vesicles is increased and the negative deviation of the plot of $1/(k_w - k_{\text{obs}})$ versus $1/[DCP]$ reflect a decrease in the amount of cytochrome b_5 in its native conformation and reduction of haem species other than native cytochrome b_5 .

When cytochrome b_5 is added to positively charged mixed DMPC/stearylamine (SA) vesicles buffered at pH 8.1 a marked increase in turbidity is observed, which suggests that vesicle fusion is occurring. The Soret peak of the cytochrome shifts by less than 2 nm, in contrast with the large spectral changes that occur [10] when cytochrome b_5 binds to negatively charged vesicles, which indicates that the cytochrome retains its native conformation when associated with the positively charged vesicles. In 0.01 M-Tris/HCl buffer, pH 8.1, an enhancement of about 70-fold in the rate of the dithionite reduction of cytochrome b_5 bound to mixed DMPC/SA vesicles at 40 mol % SA is observed (results not shown). Rate enhancements of this magnitude are expected for the reaction between negatively charged species in the presence of a positively charged surfactant system [25] as a result of the elevated local concentration of reactants at the vesicle surface.

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