The Reduction of Haem Peptides by Dithionite. A Kinetic Investigation

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Abstract

The kinetics of the reduction of the haem undecapeptide and haem nonapeptide (produced by enzymic digestion of cytochrome *c*) by sodium dithionite are reported. In keeping with other haem proteins, SO_2^- is found to be the dominant reducing agent. Rate constants in the region $2-4 \times 10^8$ M^{-1} s^{-1} are reported and these were found to be largely independent of pH and ligation state of the haem. These data are compared with the corresponding data for *c*-type cytochromes and are discussed in terms of the Marcus theory.

Slower processes follow reduction of the central iron and are interpreted in terms of ligand and spin state changes.

Introduction

Haem undecapeptide, obtained by proteolysis of cytochrome *c*, was first characterised by Tsou more than thirty years ago [1, 2]. Subsequently, a number of authors have described additional types of haem peptide and several methods of purification [3–10]. These molecules are of interest since they contain protohaem IX covalently bound through two thioether linkages to peptide chains of varying length (Fig. 1) and thus provide a means of maintaining a haem group, soluble in aqueous media over a wide range of pH, in the absence of encapsulating protein. Furthermore, haem peptides undergo similar reactions to haemoproteins, which has led to their use as 'microperoxidases' [6, 8, 11] and as CO scavengers in experiments designed to measure rate constants for dissociation of this species (CO) from haemoglobin [12, 13]. Despite the striking functional and structural similarities of the haem peptides to haemoproteins, they have found relatively few applications as model systems for elucidating aspects of haemoprotein chemistry. The usefulness of these systems for investigating the effects of axial ligand substitution on mid-point redox potentials [14, 15] and estimating the extent to which hydrophobic and steric factors in proteins might affect the thermodynamic parameters of ligand binding phenomena

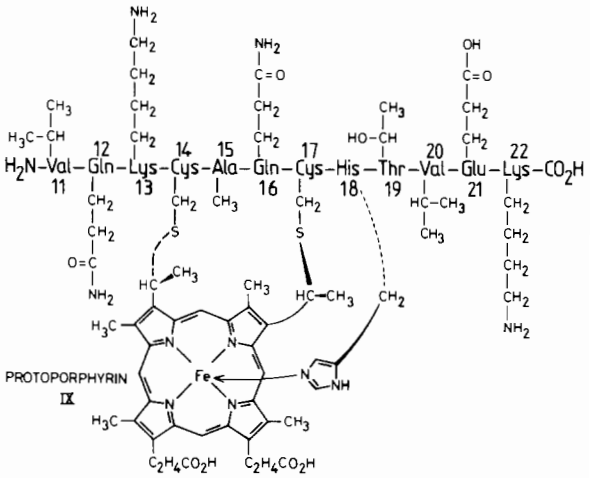

Fig. 1. Diagramatic representation of the haem peptides derived from cytochrome *c*. For simplicity, all side-chain and terminal residues are shown in their unionised forms and the counterion necessary to balance the charge on the haem has been omitted. The numbers refer to the amino acid sequence of native horse heart cytochrome *c*. Haem undecapeptide: residues 11–21; haem nonapeptide: residues 14–22.

[16, 17] have been demonstrated, but reports concerning the kinetics of haem peptide reactions have been rather few.

Under most conditions of pH, temperature, concentration and in the presence of added ligands, the fifth ligand to haem iron is undoubtedly histidine 18 (see Fig. 1) as it is in the native cytochrome. However, there is considerable uncertainty regarding the identity of the sixth ligand which appears to be variable [18]. Moreover, the sixth ligand position in haem peptides tends to be filled in an intermolecular manner, leading to oligomeric aggregates of these molecules [19]. Consequently, even reactions one might expect to proceed in a relatively simple fashion, such as cyanide binding [18] or H_2O_2 decomposition [20], unfortunately tend to exhibit polyphasic kinetics. One is therefore confronted with a potentially useful model system that is (in terms of the diversity of its reactivity) more complicated than the haemoprotein systems to which it may be applied.

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In this paper we report the kinetics of the reduction of horse heart haem undecapeptide (HUP) and haem nonapeptide (H9P) by sodium dithionite. The purpose of this work is to determine the rates of haem reduction under a variety of conditions and to compare these to the corresponding rate for native cytochrome *c*. Such a comparison is of interest in relation to theories of electron transfer in haemoproteins. In addition these experiments also yield information concerning the ligand substitution and spin state changes which occur in the peptides subsequent to a rapid change in the oxidation level of the central iron atom.

Experimental

Sodium dithionite ($Na_2S_2O_4 + H_2O$, 85% min. assay) was purchased from BDH. Unless stated to the contrary, all other reagents were of Analar grade and used without further purification. HUP and H9P were prepared from Boehringer horse heart cytochrome *c* according to previously published methods [9, 10].

Degassing of reagents was performed at room temtemperature by three cycles of evacuation and flushing with 'pure' (<5ppm O_2) nitrogen gas supplied by BOC. Reagent vessels were closed with Subaseals and transfers made under positive nitrogen pressure using syringes pre-flushed with degassed buffer. Concentrations of dithionite solutions were determined by dissolving pre-weighed solid in known volumes of buffer under anaerobic conditions, then the value of 92% suggested by Lambeth and Palmer [21] was used to calculate the dithionite concentration.

Electron absorption spectra were recorded on a Perkin-Elmer, Coleman 575 spectrophotometer. Stopped-flow measurements were made using a Durram-Gibson instrument with a deadtime of 3 ms.

Results

The absorption spectra of oxidised and dithionite-reduced HUP at pH 9.5 are presented in Fig. 2. The extinction coefficients are based on concentration determinations performed spectrophotometrically at *ca.* pH 2.0 using $\epsilon_{394} = 176–178\ mM^{-1}\ cm^{-1}$ [10, 22]. The spectral features shown are similar to those exhibited by low-spin haemoproteins and is in accord with a variety of evidence [9, 18] suggesting that at least in the ferrous form, the majority of the haem iron exists in a low-spin configuration. It should be noted that whilst in alkaline media the HUP tends to be in varying states of aggregation which are dependent upon pH, temperature and concentration [19], the spectra of Fig. 2 do not change (by more than 10%) in the following range of conditions: pH 7.5–10.5,

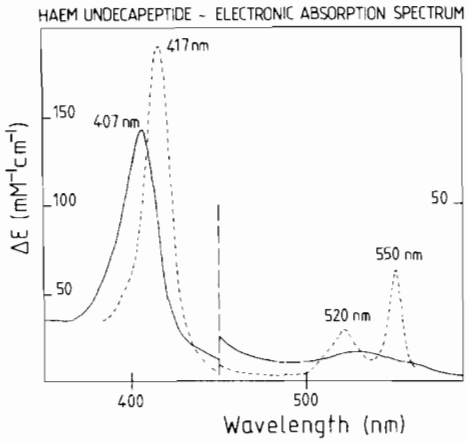


Fig. 2. Absorption spectra of haem undecapeptide: —— oxidised (as prepared); ——— reduced (excess sodium dithionite). Conditions employed: 10 μM peptide, pH 9.5 in 0.1 M sodium tetraborate buffer, 20 °C, 1 cm pathlength.

15–25 °C, 5–50 μM. For purposes of estimating concentrations of H9P solutions, we have assumed that the relevant extinction coefficients are similar to those for HUP.

It is clear from the absorption spectra of Fig. 2 that the reduction kinetics of haem peptides may be followed in the stopped-flow apparatus by making observations at around 400 nm (the Soret region) or at around 550 nm. Both types of experiment were performed. However, we found it most convenient to work in the Soret region where the larger optical density changes were advantageous in distinguishing between the various phases of the reactions observed. This was subsequently proven to be quite important since on mixing dithionite with HUP or H9P we observed multiphasic progress curves. The form of these curves was wavelength dependent. The time courses could be separated into their constitutional parts and Fig. 3 shows the kinetic difference spectrum of each of the four phases seen at pH 6.5 on reduction of HUP. In the case of the reduction of this peptide by dithionite (in the presence and absence of added cyanide) the number and direction of absorbance changes of the various phases detected in the pH range 6 to 9.5 have been documented (Table I). Phases II, III and IV were independent of dithionite concentration and consequently, are not electron transfer processes. We present the rate constants associated with these phases under a variety of conditions of pH in Table II.

Lambeth and Palmer have shown [21] that the radical SO_2^-, formed by dissociation of $S_2O_4^{2-}$, is the major reducing species present in dithionite solutions. Furthermore, under pseudo-first order conditions, reduction of oxidant by dithionite proceeds according to the rate equation:

$$k_{obs} = k_2 K^{1/2} [S_2O_4^{2-}]^{1/2} + k_1 [S_2O_4^{2-}] \quad (1)$$

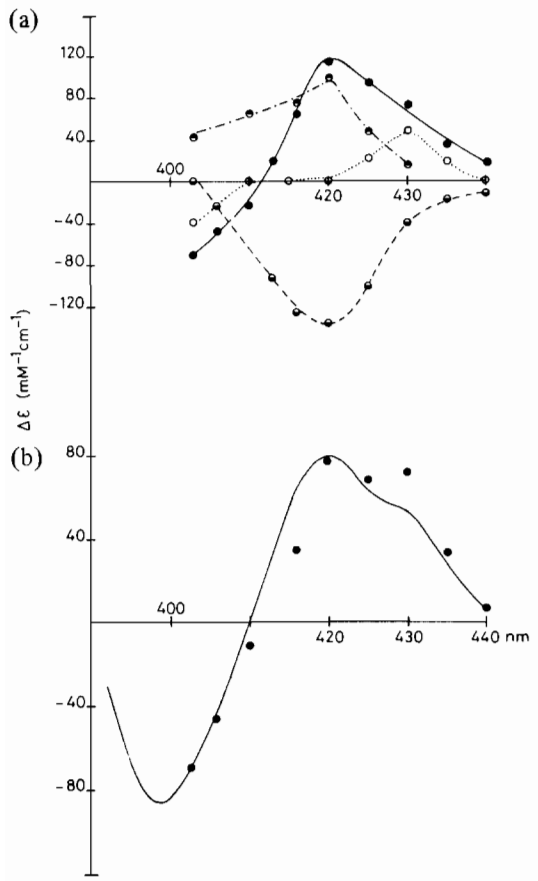


Fig. 3. (a) Kinetic difference spectrum obtained on reduction of HUP with sodium dithionite. Absorption changes associated with the four phases of the reduction of haem undecapeptide by dithionite [0.1 mM]: —— phase I; ····· phase II; ——— phase III; —·— phase IV. Conditions employed: 2.5 μM peptide, pH 6.5 in 0.1 M sodium phosphate buffer, 25 °C, 2 cm pathlength. (b) Comparison of static and kinetic difference spectra. Solid line, reduced (sodium dithionite) minus oxidised (as prepared); ●, points calculated by summation of the four phases of the kinetic difference spectra. Conditions employed: 5.0 μM peptide, pH 6.5 in 0.1 M sodium phosphate buffer, 25 °C, 1 cm pathlength.

TABLE I. Phases Observed at 430 nm During the Reduction of HUP by Dithionite

pH	$[CN^-]$ (M)	Phases (sense of absorbance change (±))[a]			
		I	II	III	IV
6.0	0.0	+	+	–	+
6.5	0.0	+	+	–	+
7.0	0.0	+	/	–	+
8.2	0.0	+	–	–	(–)
8.7	0.0	+	–	(+)	(–)
9.3	0.0	+	–	(+)	(–)
6.1	9.0×10^{-3}	+	+	–	+
7.0	2.0×10^{-3}	+	/	–	+
8.2	1.5×10^{-3}	+	/	/	(–)
8.8	1.5×10^{-3}	+	/	/	(–)
9.4	1.5×10^{-3}	+	/	/	/

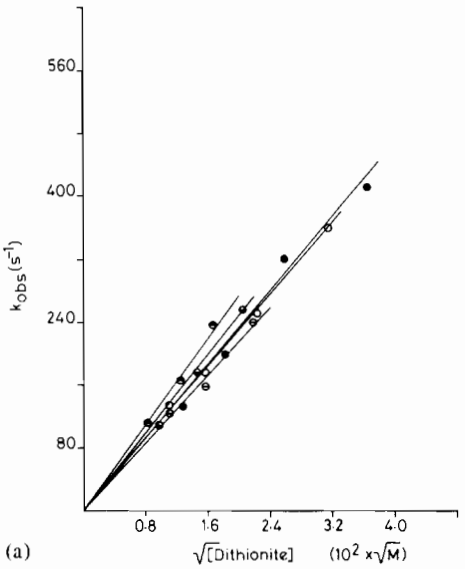
[a]+ Increase in absorbance; (+) small increase in absorbance; – decrease in absorbance; (–) small decrease in absorbance; / phase not observed.

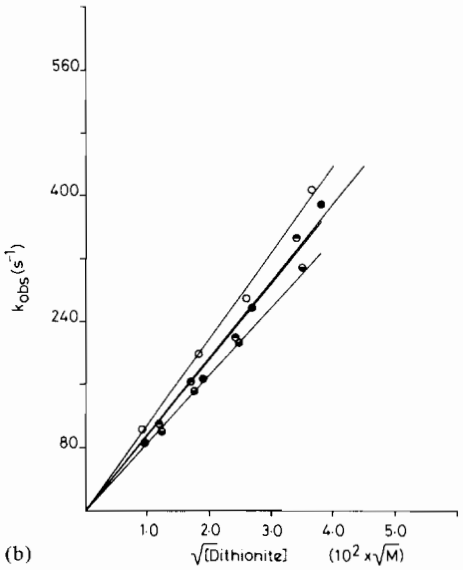
where K is the equilibrium constant for the dimer–monomer equilibrium. The term involving $[S_2O_4^{2-}]$ is only important at higher concentrations of dithionite which could not be used in haem peptide experiments since the reductions were then too fast to observe. Therefore, plotting k_{obs} (ordinate) against $[S_2O_4^{2-}]^{1/2}$ (abscissa) for the reduction of haem peptides by dithionite was found to generate straight lines passing through (or close to) the origin (Fig. 4). Moreover, these straight lines all have slope $k_2K^{1/2}$ where k_2 is the second order rate constant governing haem peptide reduction by SO_2^-, which may be readily determined provided an independent evaluation of K is available. Using the mean of the published values [21, 23], K was taken to be 1.6×10^{-9} M ($K^{1/2} = 4 \times 10^{-5}$), values for k_2 have been calculated from the data in Fig. 4 (see Table III). Our results on the reduction of the nonapeptide agree very well with those of Kazmi *et al.* [24].

TABLE II. Observed Rate Constants for the Slower Phases of the Reaction between HUP and Dithionite

pH	$[CN^-]$(M)	k_{obs} (s^{-1})[a]		
		Phase II	Phase III	Phase IV
6.0	0.0	15 (+/–5)	p	p
6.5	0.0	40 (+/–15)	1.0 (+/–0.2)	p
7.0	0.0	a	0.7 (+/–0.2)	1.3 (+/–0.4) $\times 10^{-1}$
8.2	0.0	27 (+/–7)	0.7 (+/–0.3)	1.3 (+/–0.4) $\times 10^{-1}$
9.3	0.0	11 (+/–3)	p	p
6.1	9.0×10^{-3}	30 (+/–7)	0.22 (+/–0.1)	p
7.0	2.0×10^{-3}	33 (+/–3)	0.22 (+/–0.1)	3.5 (+/–0.4) $\times 10^{-2}$
8.2	1.5×10^{-3}	a	a	2.3 (+/–0.5) $\times 10^{-2}$

[a]a Phase absent; p phase present, but k_{obs} not measured. Errors indicated in parentheses are *standard errors.*





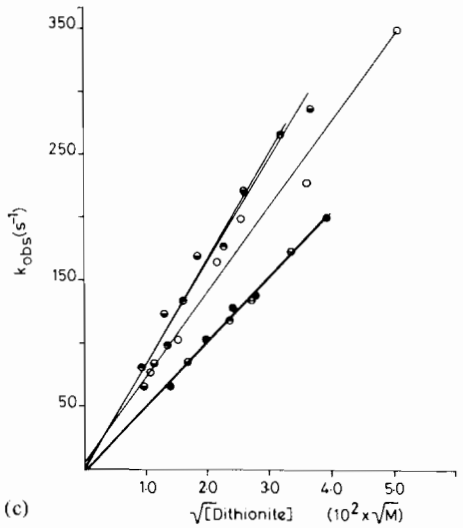


Fig. 4. Plots of k_{obs} (ordinate) *versus* $[S_2O_4^{2-}]^{1/2}$ (abscissa) for the haem peptide reduction processes (phase I). (a) Haem undecapeptide; ◓ pH 6.0; ◒ pH 6.5; ○ pH 7.0; ● pH 8.2; ⊖ pH 9.3. (b) Haem undecapeptide-cyanide; ○ pH 7.0; ● pH 8.2; ◓ pH 8.8; ◒ pH 9.4. (c) Haem nonapeptide; ◓ pH 5.9; ◒ pH 6.5; ○ pH 6.9; ⊖ pH 7.4; ● pH 7.8. Conditions employed: 2–3 μM peptide concentrations, 0.1 M sodium phosphate buffers, 25 °C.

TABLE III. Rate Constants for the Reduction of Haem Peptides by the Dithionite Radical

pH	(a) HUP	k_2 (M^{-1} s^{-1}) × 10^{-8} (b) HUP–CN^- [a]	(c) H9P
5.9			2.2 (+/−0.2)
6.0	3.6 (+/−0.4)		
6.5	3.2 (+/−0.5)		2.3 (+/−0.2)
6.9			1.6 (+/−0.2)
7.0	2.9 (+/−0.4)	2.8 (+/−0.2)	
7.4			1.3 (+/−0.1)
7.8			1.3 (+/−0.1)
8.2	3.0 (+/−0.3)	2.4 (+/−0.4)	
8.8		2.3 (+/−0.1)	
9.3	2.7 (+/−0.3)		
9.4		2.6 (+/−0.2)	

[a]Cyanide concentrations were as indicated in Tables I and II. Errors indicated in parentheses are *standard errors*.

Discussion

The Reduction Process

It is clear from the results (Table III) that the second order rate constants for the reduction of haem peptides by SO_2^- do not vary much over the range of conditions studied. At *ca.* 2×10^8 M^{-1} s^{-1}, these systems are within a couple of orders of magnitude of being rate limited by diffusion processes and are somewhat faster than the analogous cytochrome *c* reaction (3.9×10^7 M^{-1} s^{-1} at pH 8.0 [21]).

The degree of aggregation of the peptides varies considerably under the experimental conditions employed, with the 6th ligand to haem iron being CN^-, H_2O, or an amino group provided in an *intermolecular* manner [18]. Consequently, the extent to which reactant SO_2^- is hindered from approaching the most accessible axial position of the haem was

varied dramatically, with apparently little effect on observed reaction rates (Table III). Whilst the degree of total haem exposed directly to solvent/reactant may have changed from one set of conditions to another, the same is almost certainly not true for the haem edge. The size of the haem peptides is not so large that upon oligomerisation the haems become entirely surrounded by organic material. Most probably only the two faces of the haem will be sterically hindered from approaching reactant. Therefore, the lack of variation in the data is consistent with an outer-sphere mechanism of electron transfer, the haem edge being the electron entry site. The fast rates observed for the SO_2^- reactions with the peptides also favour this interpretation, since substantiated cases of inner-sphere mechanisms tend to be rather slower [25].

The most comprehensive treatment of outer-sphere reactions has been given by Marcus [26] in the form of a relatively simple relationship between the rates of self-exchange reactions ($\Delta G^\circ = 0$) and the rates of electron transfer reactions accompanied by a net chemical change ($\Delta G^\circ \neq 0$):

$$\ln k_{12} = 0.5 \left(\ln k_{11} + \ln k_{22} + \frac{2.303\ \Delta E^\circ_{12}}{0.059} + \ln f_{12}\right) \quad (2)$$

where k_{12} and the term in ΔE°_{12} are the rate and equilibrium constants for an electron transfer reaction accompanied by chemical change and k_{11} and k_{22} are the appropriate self-exchange rate constants. The correction factor, $\ln f_{12}$, is given by:

$$\ln f_{12} = -(w_{12} + w_{21} - w_{11} - w_{22})/2RT$$

where w_{12} and w_{21} refer to the work required to being the reactants or products from infinite separation to the interaction distance in the activated complex; w_{11} and w_{22} being the corresponding work terms for the self-exchange reactions.

We cannot apply eqn. (2) to the current set of data since the self-exchange rate of SO_2^- is unknown, but by making the assumption that this parameter and the appropriate redox potential do not vary significantly in the two experiments, it is possible to calculate the predicted *difference* between the rates of reduction of ferricytochrome *c* and ferric haem peptides by the dithionite radical.

Using the value of 1×10^7 M^{-1} s^{-1} for the haem peptide self-exchange rate [27] (a similar value has been reported for the haem octapeptide [28]) and -0.2 V as the mid-point potential [14], then application of eqn. (2) yields:

$$\ln k_{12} = 0.5 \left[\ln 10^7 + \ln k_{22} + \frac{2.303(E_2^\circ - 0.2)}{0.059} + \ln f_{12}\right]$$

Similarly, using the previously reported values for the self-exchange rate for horse heart cytochrome *c* of 1×10^3 M^{-1} cm^{-1} [29] and +0.2 V for the reduction potential [30], one obtains:

$$\ln k_{12} = 0.5 \left[\ln 10^3 + \ln k_{22} + \frac{2.303(E_2^\circ + 0.2)}{0.059} + \ln k_{12}\right]$$

Subtracting these two expressions (first minus second) gives:

$$\Delta \ln k_{12} = -3.2 + \Delta \ln f_{12}/2 \quad (3)$$

That is to say, ignoring the work terms ($\Delta \ln f_{12}/2$), the rate of haem peptide reduction by the dithionite radical is predicted to be some 25 times *slower* than the analogous reaction with cytochrome *c*. In fact, the measured peptide reaction rate is approximately 5 times *faster* than that of the cytochrome [21]. A comparison between the dithionite reduction rates for the peptides and cytochrome c_{551} (*Ps. aeruginosa*) may also be made. Taking the self-exchange rate of c_{551} to be $\sim 10^7$ M^{-1} s^{-1} [31] calculations (ignoring work terms) suggest that this protein should be reduced some 2000-fold more rapidly than the peptides. In fact the measured rate for the peptides is approximately 10-fold greater than that for cytochrome c_{551} (3×10^7 M^{-1} s^{-1}) [32].

The most noteworthy feature of the above data is not that the ratio of the two calculated rates expressed logarithmically in eqn. (3) is in error, but that the sign is wrong, *i.e.* the rate of cytochrome *c* reduction by SO_2^- is incorrectly predicted to be faster than that of haem peptides.

Is is known that the rate of electron transfer between cytochrome *c* and small inorganic molecules is dependent upon the relative charges on the reacting species. In particular, negatively charged reductants and oxidants transfer electrons to and from cytochrome *c* some two to three orders of magnitude faster than positively charged reactants [33]. This can be readily understood because cytochrome *c* is positively changed below pH 10 [34] and will therefore experience a net coulombic attraction for negatively changed species. If this is taken into account in the Marcus theory and the relevant evaluation of the work terms is performed, then improved agreement between measured and predicted rates is generally found [35].

On the basis of the above considerations, we may expect the measured rate of electron transfer between SO_2^- and cytochrome *c* to be faster than would be predicted by the simple Marcus theory. Haem peptides on the other hand have isoelectric points around pH 5 [9, 10] and consequently, in the range of conditions we have employed will be negatively charged. Thus, the application of the full Marcus theory to the data presently under consideration leads to a prediction of the difference between the rates of cytochrome *c* and haem peptide reduction by SO_2^- which is even more in error than that expressed in eqn. (3).

There is some evidence in the current data that increasing the net negative charge on the peptides results in a decrease in the rate of reduction by SO_2^-. For example, the HUP rates are lowered by the binding of cyanide (*c.f.* Tables IIIa and IIIb) and the H9P rate is lowered on increasing the pH (Table IIIc). These trends are not dramatic considering the range of conditions employed and tend to suggest that the total charge is perhaps not as important as the charge *distribution* on the reacting species.

The Slower Phases

Following the initial reduction of haem peptides by SO_2^- a number of slower phases may occur, depending upon the particular peptide and conditions employed. These are all dithionite concentration independent (Table II) and are therefore not reduction processes. Moreover, in the presence of a suitable excess of cyanide and at high pH, the additional phases are scarcely observed (Table I). Consequently, the slow processes may be readily interpreted as ligand substitution reactions, reflecting the varying affinity of haem iron for given ligands in its different oxidation states.

That phase which we have labelled II (Fig. 3) is only observed in the case of the HUP reactions and changed sign as a function of pH, having an apparent pK at *ca.* pH 7.0 (Table I). We ascribe this phase to ligand exchange involving the α-amino group of the N-terminal valine and the ε-amino group of lysine 13 (Fig. 1). These ligands bind to haem iron in an intermolecular manner [18] and since the exchange of one for the other is reasonably fast (Table II), this must occur without a change in the degree of polymerisation of the system. Amino acid residues 11–13 are absent from H9P (Fig. 1), thus explaining the absence of phase II from the reactions involving this peptide.

Phases III and IV are much slower than the preceeding ones (Table II) and are observed in all cases except where cyanide remains bound following reduction (Table I). Phase III is always markedly negative if the reaction is followed in the stopped-flow apparatus at *ca.* 550 nm (data not shown) in the pH range 6.5 to 10.5. This indicates that phase III corresponds to low-spin to high-spin conversion of ferrous haems. That is, displacement of intermolecularly bound secondary amino groups by water (presumably), leading to depolymerisation of the peptide aggregates. The subsequent phase IV seems to nearly always be opposite in sense to phase III (Table I). It is therefore tempting to suggest that phase IV represents a repolymerisation of the system. This is conceivable, if following phase III, a conformational change in the peptide may occur which results in an effectively different 'monomeric unit' with changed ligand donor-acceptor properties. Unfortunately, this remains speculative and we cannot exclude the possibility that other processes, such as changes in aggregation not mediated by ligand binding to the metal centre, are responsible for the finally observed phase.

Concluding Remarks

One may argue that the unexpectedly fast rate of haem peptide reduction by SO_2^- relative to that of cytochrome *c* can be understood simply in terms of an increased degree of haem edge exposure in the former system(s). However, the failure of the full Marcus theory to yield an improved prediction of the relative reaction rates over the simple theory implies that other factors are of importance in adequately accounting for the discrepancy. In particular, our data suggest that net charge on the reacting species is less important than relative charge distribution on the reactants.

The presence of a number of lysine residues around the proposed electron entry site of cytochrome *c* continues to be stressed in relation to theories of electron transfer with its *in vivo* redox partners [e.g. 36, 37] and at modified electrode surfaces [38]. Furthermore, in the case of ferricytochrome *c* reduction by $Fe(CN)_6^{4-}$ and closely related species, this area of the protein is generally agreed upon [39–41] as the target for incoming reductant. Once the reactants are spatially close, the controversy surrounding subsequent events is two-fold. Firstly, whether binding of reductant to the protein prior to electron transfer is required or not [*cf.* 42, 43] and secondly, whether haem edge contact or tunneling mechanisms prevail [*cf.* 31, 43].

The present results are of relevance to these questions since they may be interpreted in terms of different electron transfer pathways existing in the haem peptides and cytochrome *c*. Given that the SO_2^- reduction of the peptides is fast and that there is minimal steric hindrance to approach of the radical to the haem edge, it seems entirely likely that an edge contact mechanism is the case. However, the haem edge in cytochrome *c* is sterically hindered to a considerable extent [33] and the incoming negatively changed SO_2^- is likely to approach sites similar to those favoured by $Fe(CN)_6^{4-}$ [44]; *i.e.* one or more of the positively charged lysine residues which are around the haem pocket, but not immediately at the haem edge. Thus, with the protein it appears that negatively charged dithionite, although possibly being attracted to the general vicinity of the haem site by the surrounding lysine residues, finds it difficult to penetrate this region and approach closely the haem edge. Alternatively, the incoming dithionite may bind at a site even more remote from the haem edge. In either case electron transfer would occur over a

greater distance and hence more slowly than with the peptides.

Although the distance between radical and haem iron may be 12–15 Å [44], electron transfer can still be facile [45]. Nevertheless, the rate achieved will almost certainly be slower than if edge contact were involved. Under such circumstances, it is entirely reasonable that the Marcus theory does not account for the relative reduction rates of the peptides and the protein by the dithionite radical.

Finally, it should be noted that these data appear to refute the suggestion that cytochrome *c* may have a redox centre which is peculiarly poised in such a manner as to facilitate rapid electron transfer [e.g. 46, 47]; since in the absence of the protein there can be no 'entatic state' and yet the electron transfer rate has increased significantly.

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