FREE RADICAL REDUCTION OF FERRICYTOCHROME-C

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SUMMARY

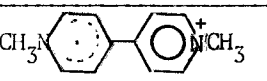
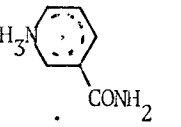
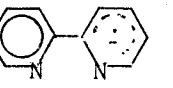
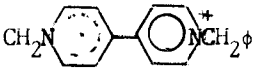
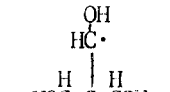
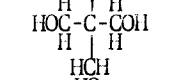
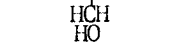
Rate constants have been determined for the reduction of ferricytochrome-c by free radicals. k values for pyridinyl radicals, benzoate adduct radicals, nitrobenzene and nitrobenzoate anion radicals, α-hydroxy radicals, formyl anion radical, and superoxide radical show no apparent correlation with size, charge, or structure of the reductant. Some k values change as the pH is increased. The methyl viologen radical is the most reactive; NAD· is reactive; the pentaerythritol radical is unreactive at pH=7 but moderately reactive at pH=9; and $O_2^{\cdot-}$ becomes less reactive with increasing pH. The results indicate that several mechanisms for electron transfer are operative and that conformers of differing reactivity influence the kinetics in alkaline solutions.

Of considerable importance to understanding biochemical electron transport is information on the mechanism and kinetics of the intriguing elementary redox reactions occurring between and within mitochondrial components. Cytochrome-c (1) is one such component under active investigation. Much of the information concerning the mechanism by which electrons are transferred to and from the heme-bound iron has come from stopped-flow investigations of various redox reactions (1). More recently, pulse radiolysis, which has the advantage of much shorter time resolution (~1μs), has also been used (2, 3, 4, 5). We have used this technique to generate various free radicals and to investigate the kinetics of their reactions with ferricytochrome-c (cyt(III)-c).

MATERIALS AND METHODS

A 13 Mev linear accelerator providing 0.2 μsec long and 50 ma peak current electron pulses, which deposit about 400 rads/pulse in the sample cell, was used to generate an initial concentration of free radicals of about $3X10^{-6}$M. Cyt(III)-c (Sigma type VI), without being purified any further, was dissolved in solutions deaerated by bubbling with pyrogallol-scrubbed, extra pure N_2. The solution to be irradiated was transferred to a 1 cm o.d., 2 cm long optical cell mounted to the detection system. Decay of the transient species generated or formation of the stable compounds produced was monitored using kinetic spectrophotometry. Most of the rate constants determined for the free radical reduction of cyt(III)-c were derived from recorded increases in absorbance at 550 nm, corresponding to the formation of ferrocytochrome (cyt(II)-c); those determined

Table 1. Rate Constants for Reduction of Cyt(III)-c by Free Radicals at 20±2°C.*

Parent Compound	Concn, M	Formate Concn, M	Gas	pH	Radical, RH	K(·RH+cyt(III)-c) M^{-1} s^{-1}
Methyl viologen	$1X10^{-3}$	0.1	N_2O	7		$2.2X10^{9}$
				10.8		$3.4X10^{8}$
Benzoate	$2X10^{-3}$		N_2	7	$[\phi CH_2CO_2H]^{\cdot}$	$1.8X10^{9}$
			N_2O	7	$OHC_6H_4CH_2CO_2H$	$<10^{6}$
1-Methyl nicotinamide	$1.5X10^{-3}$	0.1	N_2O	7		$1.4X10^{9}$
p-Nitrobenzoate	$2X10^{-3}$		N_2	6.85	$^{-}CO_2\phi\dot{N}O_2^{-}$	$9.8X10^{8}$
Acetophenone	$1X10^{-2}$		N_2	7	$\phi\dot{C}OHCH_3$	$8 X 10^{8}$
NAD^{+}	$1X10^{-3}$	0.15	N_2O	7	NAD·	$7.4X10^{8}$
Formate	$2\text{-}5X10^{-2}$		N_2O	7	$\cdot CO_2^{-}$	$6.9X10^{8}$
				10.8		$2.5X10^{8}$
2,2'-Bipyridine	$1X10^{-3}$		N_2	7		$5.7X10^{8}$
Benzyl viologen	$1X10^{-3}$	0.15	N_2O	7		$4.3X10^{8}$
i-Propanol	$5X10^{-2}$		N_2O	7	$CH_3\dot{C}OHCH_3$	$3.8X10^{8}$
	2		N_2O	7		$1.3X10^{8}$
	$5X10^{-2}$		N_2O	9.3		$1.6X10^{8}$
Lactate	$2X10^{-1}$		N_2O	7	$CH_3\dot{C}OHCO_2^{-}$	$2.3X10^{8}$
				11	$CH_3\dot{C}O^{-}CO_2^{-}$	$2.5X10^{8}$
Nitrobenzene	$5X10^{-3}$		N_2	7	$\phi\dot{N}O_2^{-}$	$2.4X10^{8}$
				9.3		$9.2X10^{7}$
Tartrate	$2X10^{-2}$		N_2O	7	$^{-}O_2CCHOH\dot{C}OHCO_2^{-}$	$1.7X10^{8}$
FMN	$1X10^{-3}$	0.1-0,4	N_2O	7	FMN·	$1.5X10^{7}$
Glycerol	$5X10^{-3}$		N_2O	7	$\cdot CHOHCHOHCH_2OH$/ $CH_2OH\dot{C}OHCH_2OH$	$2.5X10^{6}$
O_2	$1X10^{-3}$	0.15	O_2	7	$O_2^{\cdot-}$	$2.4X10^{6}$
				9.3		$1.5X10^{5}$
Pentaerythritol	$5X10^{-2}$		N_2O	5.6		$<10^{6}$
				9.1		$1.4X10^{8}$
				9.8		$1.6X10^{8}$

*Solutions were $2\text{-}4X10^{-5}$ M in cyt(III)-c and $8\text{-}10X10^{-4}$ M KH_2PO_4

for reduction by viologens were derived from the decay of these semiquinones, as monitored at 600 nm.

RESULTS AND DISCUSSION

The irradiation of water leads to the formation of primary radicals and molecular products:

$$H_2O \rightsquigarrow e^-_{aq}\ (2.8),\ OH\cdot(2.8),\ H\cdot(0.6),\ H_2O_2(0.7),\ H_2(0.4),$$

the absolute yields (number formed per 100 electron volts of energy absorbed) being shown in parentheses (6). In the presence of specific solutes these radicals can be converted quantitatively into free radicals of interest, if the conditions are chosen to satisfy various kinetic requirements. Most of these interconversion reactions are well understood, so detailed descriptions will not be given. The radicals investigated and their rate constants for reduction of cyt(III)-c are listed in Table 1, arranged approximately in order of decreasing reactivity at pH=7.

Table 1 shows that, despite the generally high reactivity of free radicals, the rate constants (k) determined here for ferricytochrome reduction at pH=7 range over about three orders of magnitude and show no clear correlation with molecular size, charge state, or oxidation potential. Some of the k values, particularly those associated with aromatic or heterocyclic ring structures, are greater than $10^9\ M^{-1}s^{-1}$. Many fall between 10^8 to $10^9\ M^{-1}s^{-1}$, while a few are well below $10^8\ M^{-1}s^{-1}$ and as low as $10^6\ M^{-1}\ s^{-1}$. Of the k values examined also at high pH, most decreased to 0.5 or 0.3 the neutral pH value. The reactivity of the pentaerythritol radical, however, increased significantly in going from pH=5.6 to pH=9.1. Our values for reduction by $CO_2^{\cdot-}$ and $CH_3\dot{C}OHCO_2^-$ radicals are in good agreement with values already reported (2,5).

The pyridinyl radicals stand out as the most reactive group of reductants, with the methyl viologen radical coming closest to reacting at a diffusion-controlled rate. Molecular size and disposition of nitrogens appear to influence subtly the reactivity, as a comparison of pyridinyls from 1-methyl nicotinamide, NAD^+, benzyl viologen, and 2,2'- bipyridine indicates. Moreover, changing the pH from 7 to 10.8 significantly decreases the k for methyl viologen. These differences could reflect variations in the efficiency with which the pyridinyls bind to or interact with specific sites on the ferricytochrome-c.

A particularly striking difference in reactivity is encountered with the electron and hydroxyl adduct radicals derived from benzoate. The former readily reduces cyt(III)-c; the latter may be incapable of such reduction, k being $<10^6 M^{-1}s^{-1}$. Polarographic evidence (7) indicates that hydroxyl adducts of aromatic molecules have low oxidizability, which would account for these observations.

Comparison of k values for benzoate, nitrobenzoate, and nitrobenzene anion radicals shows a respective decrease in reactivity, suggesting a correlation between electron delocalization and reactivity. Delocalization would facitate complex formation and/or π-π orbital electron transfer, whereas localization on the nitro group would limit the possibilities for interaction with cyt(III)-c.

Despite the commonality of a hydroxy group α to the unpaired electron in the radicals formed from acetophenone, isopropanol, lactate, tartrate, glycerol, and pentaerythritol, their k values differ by more than a factor of 400. Differences in the charge on the ions appear to have no significant effect on the reactivity. Note also that there is no pH effect on k for lactate even though at pH=11 the radical is completely deprotonated (pK_a=9.8 (8)) and ferricytochrome exists in other conformations (9,10,11). However, at pH=7 in the presence of high alcohol concentration, which is known to alter the cyt(III)-c conformation, the k for isopropanol radical decreases to a value close to that obtained at pH=9.3. The presence of the phenyl group, however, strongly influences the reactivity. Perhaps the only trend readily discernible is the decrease in reactivity as the number of hydroxyl groups increases. The radical from pentaerythritol, a tetrahydric, tetrahedrally structured alcohol, has the smallest k of this group. Moreover, it is the only radical studied whose reactivity is enhanced at relatively high pH, but not high enough for deprotonation to occur (pK_a=11 (12)).

A significant decrease in reactivity as the pH is increased is observed for the methyl viologen radical, $CO_2^{\cdot-}$, isopropanol radical, nitrobenzene anion radical, and superoxide radical ($O_2^{\cdot-}$). This effect could be related to the drop in $E^{O'}$ with pH (13) and/or the existence above pH=8 of at least two additional conformers of cyt(III)-c having different reactivity. (In the reduction of cyt(III)-c by ascorbic acid (9), there is a slow kinetic component whose contribution changes with pH and shows inflection points at pH=8.5, 9.2, and 9.8 that have been attributed to pK's for conformer equilibria.) Values of k for cyt(III)-c reduction by $CH_3\dot{C}OHCH_3$, $NO_2^{\cdot-}$, and $O_2^{\cdot-}$ have been determined as a function of pH over as wide a range as is practical. The results for $O_2^{\cdot-}$, which are representative of (but not identical to) results for the other radicals, are shown in Fig.1. k drops to half the neutral pH value at pH≅7.5 for $CH_3\dot{C}OHCH_3$ and $O_2^{\cdot-}$ and at pH≅9 for $\phi NO_2^{\cdot-}$. Preferential reaction of these radicals with the more acidic conformer (or conformers) coupled with slow conversion of the basic to the acidic conformer could account for the shape of the curve in Fig. 1.

It is interesting to compare the reactivity of a specific radical towards cyt(III)-c with its reactivity towards the bare or more accessible iron in other porphyrin compounds. At pH=7 the k value for reduction by $CO_2^{\cdot-}$ is 6.9X $10^8 M^{-1} s^{-1}$ in the case of cyt(III)-c, whereas it is 1.2X10^9 M^{-1} s^{-1} for

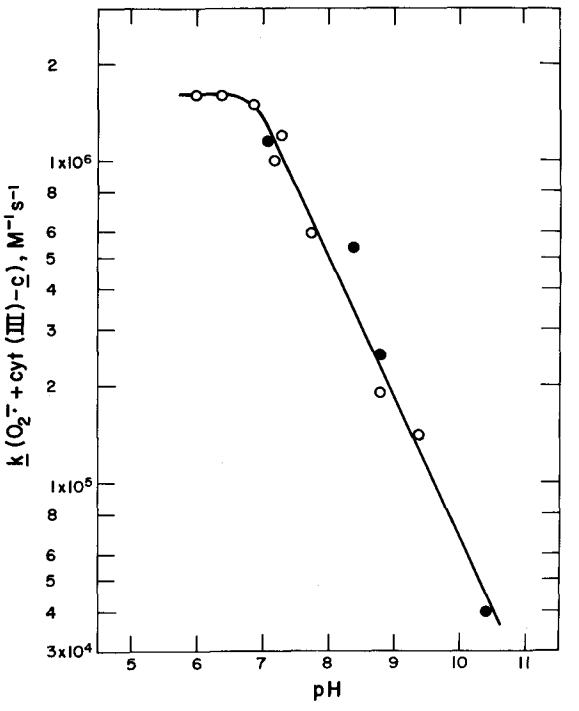


Figure 1. Effect of pH on the rate constant for the reduction of cyt(III)-c by $O_2^{\cdot-}$ radical. The filled circles correspond to solutions made from a separate lot of cyt(III)-c. All solutions were approximately 1mM in NaH_2PO_4, 0.15 M in HCO_2Na, $1.3X10^{-3}$ M in O_2, and $2X10^{-5}$ M in cyt(III)-c, which was added after saturating with O_2. The dose per pulse was kept as low as was practical, particularly as the pH was increased, to avoid any contribution to the $O_2^{\cdot-}$ decay by radical-radical reaction.

hemin(III)-c (14), which has a simple heme prosthetic group, and ~$2X10^9$ M^{-1} s^{-1} for metmyoglobin (15), in which the fifth coordinated position is protein bound. Most strikingly, the k at pH=7 for reduction by the pentaerythritol radical of cyt(III)-c is $<10^6$ M^{-1} s^{-1}, yet it is $3.0X10^8$ M^{-1} s^{-1} in the case of hemin (III)-c (14). The comparison of $CO_2^{\cdot-}$ reactivities suggests that protein binding of the iron in the sixth coordinated position introduces only a small barrier to reduction by a small and reactive reductant. The comparison of pentaerythritol reactivities suggests that a large radical having an inherently lower oxidizability can still rapidly reduce a highly accessible iron(III). (The markedly higher k at pH=9-10 for the reaction of the pentaerythritol radical with cyt(III)-c suggests that the iron in the alkaline conformers is sufficiently accessible to offset partially their apparent lower reducibility.) It is interesting, however, that the methyl viologen radical has a higher reactivity towards cyt(III)-c than it does towards metmyoglobin ($k=2.3X10^8$ M^{-1} s^{-1} (15)) despite the greater exposure of the iron in the latter.

All of these results lead to the conclusion that reduction of cyt(III)-c by free radicals must proceed by more than one of the several mechanisms mentioned by Dickerson (10), Harbury and Marks (16),and Sutin (17). The large k values for the heterocyclic nitrogen radicals are consistent with a cascading electron transfer involving π-orbital overlap of aromatic moieties that is facilitated by molecular complex formation. The moderately high values for the simple isopropanol and lactate radicals conceivably correspond to reaction with the edge of the heme group that is aligned with the crevice. A small, reactive species such as $CO_2^{\cdot-}$ can be considered to enter the crevice or otherwise penetrate the protein structure and then to transfer an electron. Some of the other more complex radicals must react by an as yet unspecified tunneling mechanism or by interaction with specific groups on the surface of the protein. Moreover, changes in the detailed structure of the protein, as effected by pH or denaturing agents, clearly alter the barriers to the electron transfer processes.

Accordingly, the influence of protein conformation, iron accessibility, and disposition of other specific reactive sites on the reduction of ferriheme compounds by free radicals are being further investigated. Particular emphasis is being placed on the factors responsible for the rapid reduction of cyt(III)-c by aromatic radicals.

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