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Thiyl free radicals and the oxidation of ferrocytochrome *c*

Direct observation of coupled hydrogen-atom- and electron-transfer reactions

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Absolute rate constants for the reaction of ferrocytochrome *c* with the thiyl radicals derived from cysteine, GSH, penicillamine and *N*-(2-mercaptopropionyl)glycine were measured by using the technique of pulse radiolysis. The reaction is believed to occur through a one-electron-transfer process, in agreement with the hypothesis that thiols may act as catalysts linking hydrogen-atom- and electron-transfer reactions.

INTRODUCTION

Of the several techniques that have been employed to study the reactions of redox agents and cytochrome *c*, the technique of pulse radiolysis has proved particularly useful (Ferguson-Miller *et al.*, 1979). For example the reactions of the oxidizing free radicals $Br_2^{\cdot-}$ and $N_3^{\cdot}$ with ferrocytochrome *c* and the reactions of $CO_2^{\cdot-}$, $(CH_3)_2COH^{\cdot}$ and $O_2^{\cdot-}$ with ferricytochrome *c* have been monitored directly (Land & Swallow, 1971, 1974; Pecht & Faraggi, 1972; Butler *et al.*, 1975, 1982; Simic *et al.*, 1975; Seki *et al.*, 1976; Koppenol *et al.*, 1976; Seki & Imamura, 1981). It has also been shown that thiyl free radicals derived from thiol-containing compounds may themselves undergo subsequent electron-transfer reactions with several biomolecules, including ascorbic acid, NADH and several phenothiazines (Forni *et al.*, 1983; Wolfenden & Willson, 1982; Forni & Willson, 1986).

We now report the direct observation of the reactions of such thiyl radicals with ferrocytochrome *c*, reactions that further illustrate how thiol compounds may act as catalytic intermediates linking hydrogen-atom- and electron-transfer reactions.

MATERIALS AND METHODS

Propan-2-ol and acetone (AnalaR) were supplied by BDH Chemicals. Cysteine hydrochloride, GSH and penicillamine were obtained from Sigma Chemical Co., and were of the purest grade available; Thiola [*N*-(2-mercaptopropionyl)glycine] was a gift from Santen Pharmaceuticals. The cytochrome *c* (horse heart type III) was supplied by Sigma Chemical Co.

Pulse-radiolysis experiments were performed on the Brunel 4 MeV linear accelerator using a 200 ns electron pulse, which has been described previously (Willson, 1982). Doses of up to 10 J/kg were used, as determined by thiocyanate dosimetry. Solutions were prepared just before experimentation in Millipore-filtered distilled water and saturated with N_2 (British Oxygen Co.) by using the syringe bubbling technique. Cytochrome *c* (Cyt.*c*) was reduced by addition of the thiol under study to a stock cytochrome *c* solution; typical concentrations used were 10 mM-thiol and 1 mM-cytochrome. In the presence of O_2 the reduction was complete within 30 min, as monitored at 550 nm with a Varian Cary 219 spectrophotometer. Previous studies indicate that the reaction proceeds through thiol autoxidation (Misra, 1974); superoxide anion ($O_2^{\cdot-}$) is generated, and this in turn reduces the cytochrome with $k_1 = 2.6 \times 10^5\ M^{-1} \cdot s^{-1}$ (Butler *et al.*, 1982):

$$O_2^{\cdot-} + \text{Cyt(III)}.c \rightarrow O_2 + \text{Cyt(II)}.c \quad (1)$$

Thiyl radicals were generated in de-aerated aqueous solutions containing the thiol compound (5–50 mM), propan-2-ol and acetone (1 M) according to:

$$H_2O \rightarrow OH^{\cdot}(45\%) + e^-_{aq.}(45\%) + H^{\cdot}(10\%) \quad (2)$$

$$OH^{\cdot} + (CH_3)_2CHOH \rightarrow H_2O + (CH_3)_2COH^{\cdot} \quad (3)$$

$$H^{\cdot} + (CH_3)_2CHOH \rightarrow H_2 + (CH_3)_2COH^{\cdot} \quad (4)$$

$$e^-_{aq.} + (CH_3)_2CO \rightarrow (CH_3)_2CO^{\cdot-} \quad (6)$$

$$(CH_3)_2CO^{\cdot-} + H_2O \rightarrow (CH_3)_2COH^{\cdot} + OH^- \quad (5)$$

$$(CH_3)_2COH^{\cdot} + RSH \rightarrow (CH_3)_2CHOH + RS^{\cdot} \quad (7)$$

Experiments were performed at pH 6 unless otherwise stated; pH was adjusted with NaOH.

RESULTS

On pulse radiolysis of a solution containing 1 M-acetone/propan-2-ol, 5 mM-GSH and 50 μM-ferrocytochrome *c*, N_2-saturated at pH 7, a strong absorption that increased exponentially with time appeared at 460 nm (Fig. 1). A concomitant decrease in absorption occurred at 550 nm, in agreement with reactions (1)–(7) followed by:

$$GS^{\cdot} + \text{Cyt(II)}.c \rightarrow GS^- + \text{Cyt(III)}.c \quad (8)$$

The magnitude of the absorption change corresponded to a 96% conversion of ferrocytochrome *c* into ferricytochrome *c* at 460 nm, assuming $G = 0.6\ \mu M \cdot J^{-1}$ and $\epsilon_{460} = 6700\ M^{-1} \cdot cm^{-1}$ (Margoliash & Frohwirt, 1959).

In order to confirm that ferricytochrome *c* was formed in the above reaction, spectral analysis was undertaken after pulse radiolysis of N_2-saturated solutions (at pH 6) containing 1 M-acetone/propan-2-ol, 10 mM-GSH and 50 μM-ferrocytochrome *c* (Fig. 2). Also shown is the theoretical difference spectrum normalized to the experimental data at 480 nm assuming the yield of thiyl radicals is $0.6\ \mu M \cdot J^{-1}$. Clearly there is excellent agreement between the data apart from the α-band at 550 nm, where the high ground-state absorption of the reduced cytochrome at this wavelength ($\epsilon_{550} = 27600\ M^{-1} \cdot cm^{-1}$)

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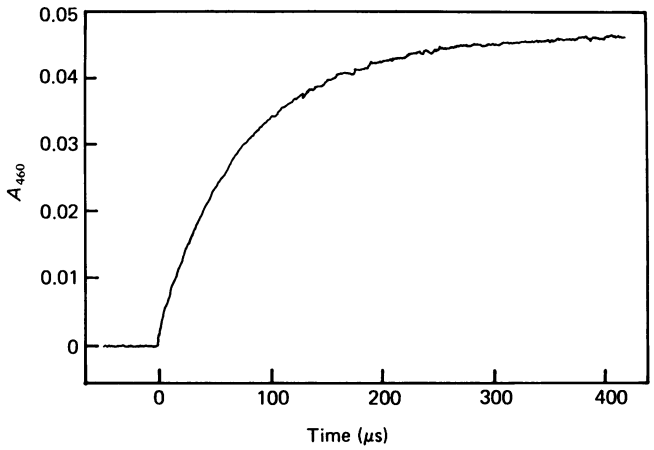


Fig. 1. Changes with time in absorption at 460 nm on pulse radiolysis of an N_2-saturated solution containing 1 M-acetone/propan-2-ol, 50 mM-GSH and 50 μM-ferrocytochrome *c* at pH 7

The dose was 8 J/kg.

(Margoliash & Frohwirt, 1959), as well as the narrow band associated with this absorption, makes accurate spectral measurement extremely difficult. No measurements were undertaken below 440 nm because of the high absorption due to the Soret band.

The spectral studies provide no evidence for the formation of any intermediate species on oxidation of ferrocytochrome *c* by glutathione thiyl radicals (GS·). To provide supporting evidence that thiyl radicals were the oxidizing species under these conditions, the effect of pH on the yield of ferricytochrome *c* was determined. De-aerated solutions of different pH values containing 1 M-acetone/propan-2-ol, 50 mM-GSH and 25 μM-ferrocytochrome *c* were studied. Increasing the pH above approx. pH 6 resulted in a decreased yield of ferricyto-

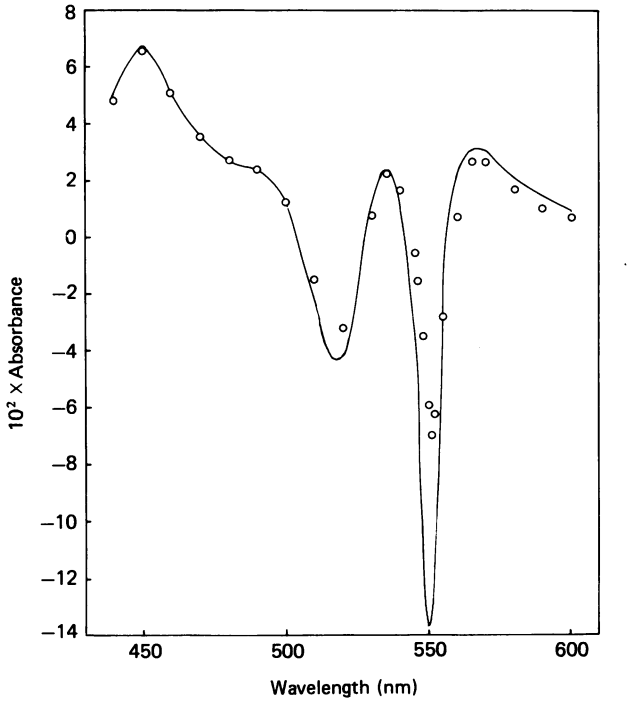


Fig. 2. Absorption spectrum recorded 800 μs after the pulse radiolysis of an N_2-saturated solution containing 1 M-acetone/propan-2-ol, 10 mM-GSH and 50 μM-ferrocytochrome *c* at pH 6

The continuous line represents the theoretical difference spectrum normalized to the experimental data at 480 nm assuming that the yield of thiyl radicals is 0.6 $\mu M \cdot J^{-1}$. The dose was 8 J/kg.

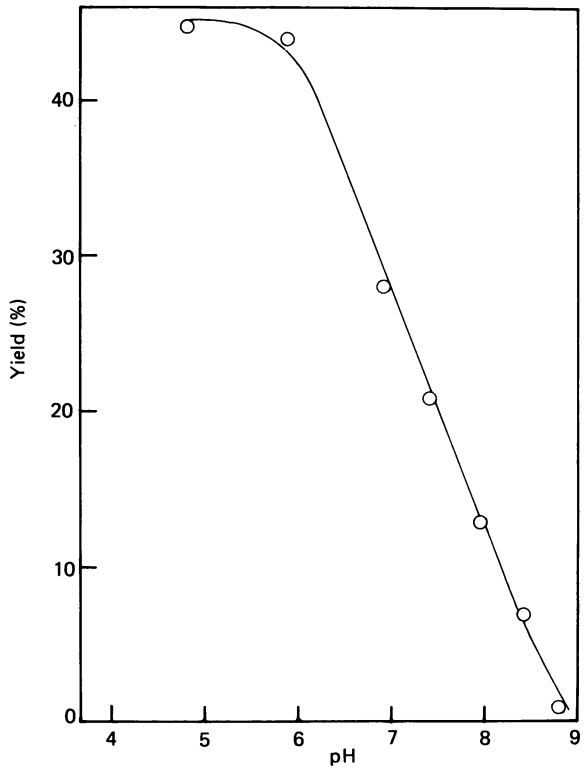


Fig. 3. Change in yield of ferricytochrome *c* as measured at 550 nm after pulse radiolysis of an N_2-saturated solution containing 1 M-acetone/propan-2-ol, 50 mM-GSH and 25 μM-ferrocytochrome *c* at pH 6

The dose was 5 J/kg.

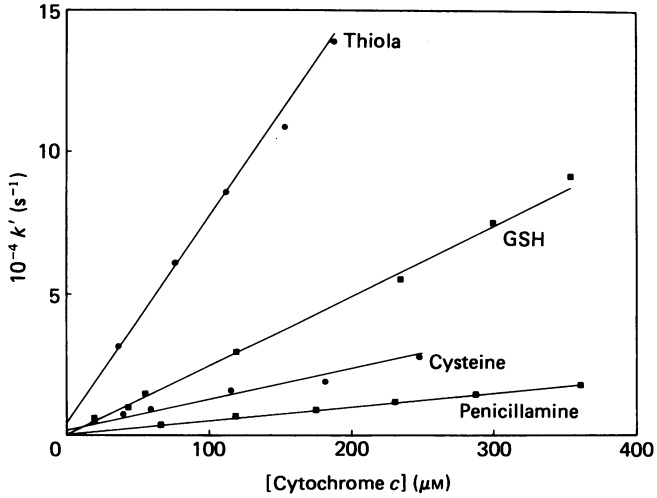


Fig. 4. Plot of first-order rate constant for the formation of the absorption at 460 nm observed on pulse radiolysis of N_2-saturated solutions of ferrocytochrome *c* containing 1 M-acetone/propan-2-ol and 10 mM-thiol at pH 6

The dose was 6–10 J/kg.

Table 1. First-order bimolecular rate constants for the reaction between thiyl radicals and ferrocytochrome *c*

The percentage yields are calculated assuming a thiyl radical yield of $0.6\ \mu M \cdot J^{-1}$ in 1 M-acetone/propan-2-ol and $\epsilon_{460} = 6700\ M^{-1} \cdot cm^{-1}$.

Thiol	$10^{-8} \times k$ ($M^{-1} \cdot s^{-1}$)	Yield (%)
Cysteine	1.1 ± 0.2	80
GSH	2.5 ± 0.2	100
Penicillamine	0.47 ± 0.05	75
Thiola	6.9 ± 0.4	100

chrome *c* at 550 nm (Fig. 3). Since thiyl radicals are formed under these conditions through reactions (1)–(7), the decreased yield can be explained by reaction (9) competing with reaction (8):

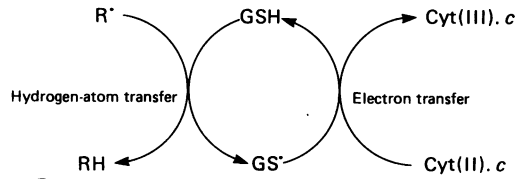
$$RS^{\cdot} + RS^{-} \rightleftharpoons RSSR^{\cdot -} \quad (9)$$

Although the disulphide radical anion $RSSR^{\cdot -}$ has a characteristic absorption at 410 nm its formation under these conditions could not be confirmed owing to overlap of the ferrocytochrome *c* absorption (Adams *et al.*, 1967). When the ferrocytochrome *c* concentration was increased, the rate of formation of the absorption at 460 nm increased. A plot of first-order rate constant against ferrocytochrome *c* concentration corresponded to $k_8 = 2.5 \times 10^8\ M^{-1} \cdot s^{-1}$. On replacing GSH with cysteine, pencillamine or Thiola, again a rapid increase in the absorption at 460 nm was found, characteristic of ferricytochrome *c*; the kinetic plots obtained are shown in Fig. 4, together with the corresponding bimolecular rate constants in Table 1.

DISCUSSION

Cytochrome *c* undergoes rapid one-electron-transfer reactions with several free-radical species. The present data indicate that ferrocytochrome *c* is rapidly oxidized by thiyl radicals and that the process occurs without the formation of any optically determinable intermediates. By analogy with previous studies (Seki & Imamura, 1981) the results indicate that direct one-electron transfer takes place at the exposed haem edge, the part of the redox centre available at the surface of the protein (Ewall & Bennett, 1974; McArdle *et al.*, 1974; Cassatt & Marini, 1974). Oxidation by the thiyl radicals derived from GSH and Thiola are 100% efficient under the conditions used, though reactions involving the penicillamine and cysteine thiyl radicals were not as efficient (Table 1). This is probably a reflection of the lower pK_a values for these thiols and therefore increased thiolate concentration at the pH used, so that consequently the likelihood of reaction (9) occurring will be greater.

Thiol-containing compounds have often been proposed as protective agents against free-radical-mediated tissue injury. Indeed, there is now considerable evidence that cells deprived of GSH, as the result of a mutation or because of the presence of exogenous agents such as diamide, are more sensitive to the damaging action of ionizing radiation (Edgren *et al.*, 1970; Harris & Power, 1973). The most common mechanism proposed for such a protective action is the repair of an initial lesion through hydrogen-atom transfer. Such reactions may also be relevant to the mechanistic role of GSH and other sulphur-containing compounds in protecting cells against free-radical-mediated injury. The observation that ferrocytochrome *c* can react rapidly with thiyl radicals but not with the propan-2-ol free radical adds further support to the concept that thiols might act as catalysts for the repair of an organic carbon-centred radical by a compound that more readily enters into electron-transfer rather than hydrogen-transfer reactions (Scheme 1).



Scheme 1. Proposed catalytic role of GSH linking hydrogen-atom- and electron-transfer reactions

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