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Kinetic Studies on the Reduction of Cytochrome *c*: Reaction with Organic Oxy-compounds

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Abstract

A range of organic hydroxy compounds, many of them naturally occurring, have been assayed for their ability to reduce the electron transfer protein cytochrome *c*. Those with conjugated hydroxyl systems e.g. catechol, acted as reducing agents while those which were phenol-like, either by separation of conjugation e.g. resorcinol or by having only one free hydroxyl group, did not. Rapid reaction kinetic investigations of the reaction of rhodizonic acid with cytochrome *c* revealed rapid reduction of the protein. The dianion of rhodizonic acid is the most reactive species in agreement with results obtained with catecholato compounds. The pH-dependence of this reaction is discussed in terms of the complex solution chemistry of rhodizonic acid.

Introduction

Dihydroxy conjugated compounds play a major role in biological redox systems and are widespread in nature. To illustrate this we may give the following examples:

(1) The quinol/quinone couple forms an important constituent of the mitochondrial respiratory chain, linking hydrogen and electron transfer components.

(2) The carbohydrate vitamin, ascorbic acid, itself a reducing agent, is ubiquitously found in eukaryotic organisms. In some bacteria other carbohydrate compounds may serve a similar function, e.g. Rhodizonic acid [1] formed from inositol.

(3) A diphenol, catecholic moiety, forms part of the bacterial iron chelator enterobactin. It has been proposed [2], that this catechol moiety, *in vitro* at least, also plays a redox role, reducting bound iron prior to iron release.

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We have previously investigated [3] the redox reactions of a wide range of substituted diphenols with cytochrome *c* and the copper protein azurin. The reactivities of these compounds could be rationalised in terms of the presence of conjugated hydroxy groups, the pK_a values of these and the resonance and inductive effects of substituents on the ring structure. In this report we extend the work to include a variety of other hydroxy compounds, in particular the oxy-carbon analogues of ascorbic acid, rhodizonic acid and squaric acid, and other hydroxy compounds both conjugated and non-conjugated.

Our results demonstrate that in order for these compounds to act as reducing agents the hydroxyl groups must be conjugated. A complete quantitative analysis of the reaction of cytochrome *c* with rhodizonic acid proved difficult due to the complex solution chemistry of this compound. Nevertheless, the results obtained with this compound are in general agreement with those obtained with ascorbic acid and catecholato compounds. In particular, it appears that it is the deprotonated forms which act as reducing agent. Although it has not proved possible to assign rate coefficients to the mono-anionic and dianionic forms of rhodozonic acid, it appears that the dianionic form is the more effective in reducing cytochrome *c*.

Experimental

Materials

Rhodizonic acid, sodium rhodizonate, hydroquinone, ascorbic acid, squaric acid and 2,3-dihydroxy pyridine, were purchased from Sigma Chemical Company (London). Phenol, catechol, maltol, resorcinol, pyrogallol were purchased from Aldrich Chemical Company (London). 2-Methoxy-phenol, 2-hydroxy benzylalcohol were purchased from Florochem Limited (U.K.). 4,3′-dihydroxy biphenyl,

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bis(4-hydroxy)phenylmethane, 4,4′-dihydroxybiphenyl and 4-hydroxybenzyl alcohol were a kind gift from Dr. J. Tillett (Essex Univerity, U.K.).

Methods

Protein concentration was determined by using the extinction coefficient of 27 600 M^{-1} cm^{-1} [4]. All experiments were performed under nitrogen unless otherwise stated. The temperature at which all experiments were carried out was 25 °C, and all experiments were performed using 0.2 M bis-tris buffer unless otherwise stated.

Stopped-flow experiments were performed in a Durrum-Gibson instrument with 2 cm light path and a dead time of 3 ms.

Procedure used to assay whether compounds were to reduce cytochrome c

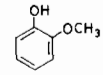
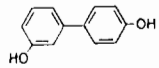
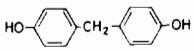
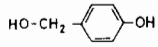
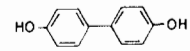
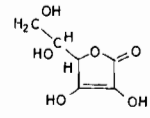
Anaerobic solutions (*ca.* 30 mM) were prepared of each of the compounds listed in Table I at pH 8 in 0.2 M bis-tris/HCl and added to anaerobic solutions of about 30 μM cytochrome *c* (pH 8) to give a reductant concentration at least 10 times greater than the cytochrome *c* concentration. The mixture was left up to 6 h. Then the spectrum of cytochrome *c* was recorded to identify whether the cytochrome *c* had been reduced.

Results

Rapid mixing experiments in which ferricytochrome *c* was mixed with rhodizonic acid gave rise to complex progress curves. The form of these curves depended both on the pH and the nature of the buffer. Examination of the wavelength dependence of the overall change in absorbance indicated that the ferricytochrome *c* was becoming reduced. The total absorbance change corresponded to that expected, thus indicating that no kinetic process was lost in the dead time (3 ms) of the apparatus.

As an example of the reduction Fig. 1 depicts the progress curves for the reduction of ferricytochrome *c* by rhodizonic acid at pH 8. At higher rhodizonic acid concentrations the progress curves are comparatively simple, whereas at lower concentrations slower phases appeared. The slow phases could not be attributed to interference from side reactions involving oxygen, as they remained even when stringent precautions were taken to ensure anaerobicity and were unaffected by controlled addition of oxygen. Likewise, this complexity was not due to multiple forms of cytochrome *c* known to exist at alkaline pH values [5] as the size and the rate of the slow phases were concentration dependent and also because similar complexity was observed at pH 7 and pH 6, where cytochrome *c* exists in a single form reducible by other hydroxy compounds [3].

TABLE I. Qualitative Comparison of some Properties of Hydroxy Compounds as Reducing Agents to Cytochrome *c*

Compound	Structure	Effect
Resorcinol		−ve
Maltol		−ve
2-Methoxyphenol		−ve
Phenol		−ve
2-Hydroxybenzyl alcohol		−ve
4,3′-Dihydroxybiphenyl[a]		−ve
Bis(4-hydroxy)phenyl methane[a]		−ve
4-Hydroxybenzyl alcohol		−ve
Squaric acid		−ve
4,4′-Dihydroxybiphenyl[a]		+ve
Pyrogallol		decomposes (+ve)
Catechol		+ve
Hydroquinone		+ve
2,3-Dihydroxypyridine		+ve
Ascorbic acid		+ve

[a]Solvent used was 30% dimethyl sulphoxide.

Although the kinetics were complex we have determined the apparent pseudo first order rate constant for the fast reaction from the initial slope of the

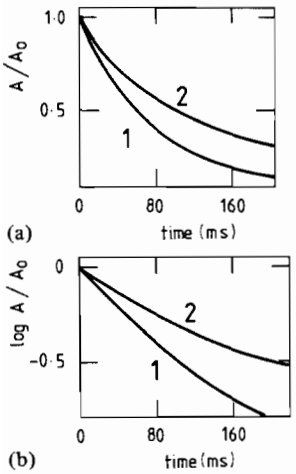


Fig. 1. Reduction of mammalian cytochrome *c* by rhodizonic acid 25 °C. Reduction was followed at 565 nm in a stopped-flow apparatus. The buffer was 0.2 M bis-tris/HCl buffer pH 8. (a) 1 and 2 show the absorbance change normalised to the overall absorbance change, *vs.* time. (b) 1 and 2 are the respective logarithmic plots. The concentration of rhodizonic acid in 1 was 2.1 mM after mixing, while it was 1.3 mM in 2. The cytochrome *c* concentration was approximately 39 μM in both experiments.

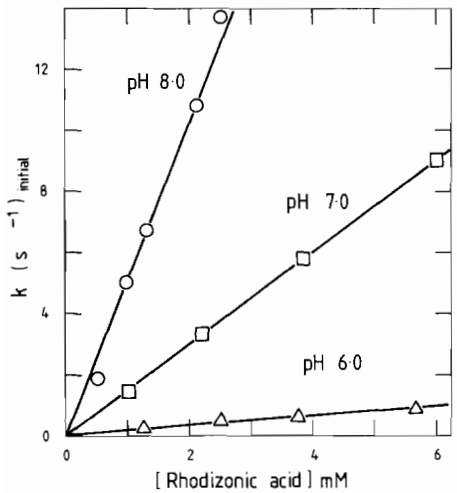


Fig. 2. Concentration dependence of the reduction of cytochrome *c* by rhodizonic acid. Buffer and temperature conditions as given in Fig. 1.

plots of the logarithm of the absorbance change *versus* time.

Figure 2 shows the dependence of this pseudo first order rate on the concentration of the reductant. This figure indicates the second order character of the reaction between cytochrome *c* and rhodizonic acid, also that the rates are proton linked. This latter point is more clearly illustrated in Fig. 3 where the logarithm of the apparent second order rate constant is plotted against pH. We have observed different kinetic behaviour for sodium rhodizonate

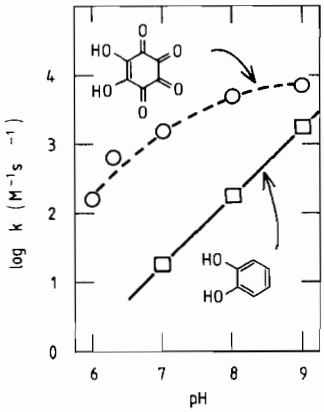


Fig. 3. The pH dependence of the apparent second order rate constant for the reduction of cytochrome *c* by rhodizonic acid (○) and catechol (□). Conditions are the same as in Fig. 1.

from that of rhodizonic acid. The latter was more soluble and reduced cytochrome *c* at a faster rate than the former. For example, at pH 7 the second order rate for the reduction of cytochrome *c* by rhodizonic acid is *ca.* 1.6×10^3 M^{-1} s^{-1}, while for sodium rhodizonate it is *ca.* 46 M^{-1} s^{-1}.

At around pH 9 a slow concentration independent phase, amounting to approximately one half of the absorbance change, was observed. This behaviour is well documented for ascorbic acid and tetrachloroquinol and arises from the existence of an 'alkaline' form of cytochrome *c* which is only very slowly, if at all, reduced by ascorbic acid [6, 7]. The slow phase of reduction is thus rate limited by the interconversion of the 'alkaline' form to that form predominating at neutral pH and with which it is in equilibrium [8]. Our findings indicate, therefore, that like ascorbic acid and conjugated dihydroxy compounds [3, 7], rhodizonic acid is unable to reduce the 'alkaline' form of cytochrome *c*.

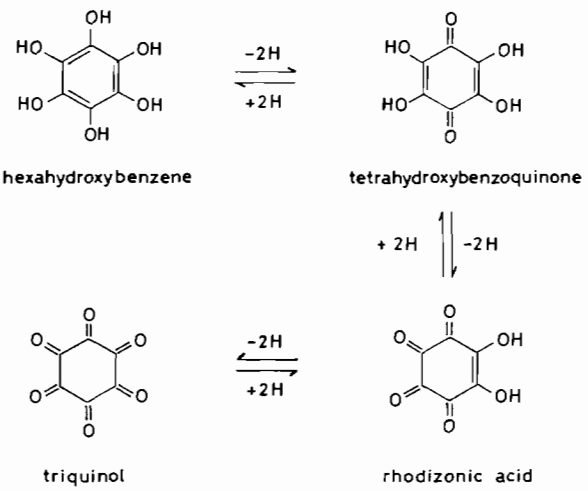
We have scanned a wide range of hydroxy compounds with regard to their ability to reduce cytochrome *c*. In Table I we give a qualitative comparison of such compounds noting solely whether they can act as reductants, without taking into consideration the kinetics of the redox process, some of which are considered elsewhere.

The pattern which emerges is that phenol and phenol-like compounds either by separation of conjugation, e.g. Resorcinol and 2-hydroxybenzyl-alcohol, or by having only one free hydroxyl group in the compound, e.g. maltol and 2-methoxyphenol, did not reduce cytochrome *c*, while any conjugated hydroxyl system did, e.g. catechol and hydroquinone. The only exception to this pattern is squaric acid. This discrepancy may be attributed to the strain in the ring system of the molecule such that oxidising the hydroxyl groups to the quinone forms is highly unfavoured.

Discussion

The reasons for the unusual behaviour of rhodizonic acid compared to other hydroxy compounds [3], may be found in its complex solution chemistry.

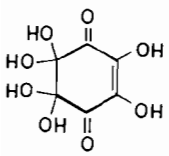
Rhodizonic acid is known to participate in a dismutation process involving tetrahydroxy benzoquinone which is dependent on many factors, e.g. pH and concentration [1, 9]. Such a process in which two molecules of tetrahydroxy benzoquinone dismute to form hexahydroxy benzene and rhodizonic acid, is depicted in Scheme 1. If an anaerobic



Scheme 1.

solution to tetrahydroxybenzoquinone at neutral pH is prepared, the deep yellow colour of rhodizonic acid is immediately observed. This indicates that part of the tetrahydroxybenzoquinone has dismutated to rhodizonic acid and hexahydroxybenzene.

In addition, rhodizonic acid undergoes a hydration process above its second pK (*ca.* 4.5), where the water of hydration becomes part of its structure (see Structure **I**) [1, 10].



I

Another complexity arises from the reported different crystalline structures of rhodizonic acids and its salts [11, 12], which may be the reason for the differences in kinetic behaviour reported above.

The aerobicity of rhodizonic acid solutions affects the rate at which it reduces cytochrome *c*. The low pK_a values of rhodizonic acid pK_{a^1} = 4.1 and pK_{a^2} = 4.5 [10] explains its reactivity towards oxygen. Thus the complex properties of rhodizonic acid seen in Fig. 3 (pH dependence of the reduction of cytochrome *c*) do not follow the simple formulation reported for the pH dependent reduction of cytochrome *c* by catechol [3]. If it did, no pH dependence should be observed for the reduction process, as the pH range explored was significantly higher than the second pK_a of rhodizonic acid. We attribute this unexpected pH dependence of the presence of many species in solution, e.g. tetrahydroxybenzoquinone, hexahydroxybenzene and the hydrated forms of rhodizonic acid. Hence, the overall effects of pH depends on the equilibrium between the different species at a given pH and temperature. This renders the task of extracting precise quantitative values very difficult. Nevertheless, as the pH range explored is significantly higher than the higher pK_a of rhodizionic acid it appears likely that it is the dianion which is acting as the reducing agent.

It is clear from Table I that phenol and phenol-like compounds are unable to reduce cytochrome *c* [13, 14] almost certainly because they are unable to form quinonic products. Polymerisation is the only way for a phenolic radical to form a product and such processes are very unfavourable [15]. Where a conjugated dihydroxy system, e.g. catechol, ascorbic acid, rhodizonic acid, etc., donates an electron to cytochrome *c* the resulting radical species may readily dismute to form the quinonic form [7, 16–18]. Disruption of the conjugation or blocking one conjugated hydroxyl group will thus render the molecule unable to reduce cytochrome *c*, e.g. 2-hydroxybenzylalcohol and 2-methoxyphenol respectively. The other compounds used lend support to this conclusion (see Table I and ref. 3).

Apart from the conjugation of the hydroxyl groups several other factors are important in controlling the electron transfer rates. As reported by Saleem and Wilson [3], the pH independent second order rates for cytochrome *c* reduction by the mono-anions of catechol and hydroquinone are 3.31×10^3 M^{-1} s^{-1} and 2.57×10^4 M^{-1} s^{-1}, respectively. This difference in reactivity may be attributed as follows.

(1) Steric factors: In catechol, the hydroxyl groups are ortho to each other, while in quinol they are para. Therefore, steric factors may play some role in determining the rate of electron transfer.

(2) Hydrogen bonding: Catechol is able to participate in both intermolecular and intramolecular hydrogen bonding [19], while the quinol can form intermolecular hydrogen bonds only.

As hydrogen bonding reduces the availability of the electrons for donation, it is reasonable to expect a difference in the rates of reduction of cytochrome *c* by these molecules and also in their auto-oxidation

properties. Aerobic catechol solutions are stable for up to 3 h and while hydroquine reacts.

(3) Ring substituents: The effects of substituents on the pK_a's of the hydroxyl groups and on the availability of electrons for transfer play or important role in determining the kinetic behaviour. This factor is fully discussed elsewhere [3].

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