Changes in Conformation of Reduced Cytochrome *c* in Neutral Aqueous Solution

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In neutral aqueous solution, high concentrations of alcohols perturb cytochrome *c* in such a way that reduction by means of ionizing radiation results in the formation of abnormal conformers of ferrocytochrome *c* resembling those previously seen on reduction in alkaline solution. These relax to normal ferrocytochrome *c* in about 0.1 s.

After cytochrome *c* has been reduced in alkaline solution it is possible to see slow spectroscopic changes near 400 nm which reflect an alteration in protein conformation, from that of the oxidized form to that of the reduced form (Land & Swallow, 1971, 1974). No such spectroscopic changes could be seen in neutral solution, although changes in conformation might be expected. Other workers, however, using different experimental conditions, have seen slow spectroscopic changes at neutral pH (Pecht & Faraggi, 1972). In view of the importance of protein conformational changes we have now made further studies of the processes.

Materials and Methods

Horse heart cytochrome *c* was from Koch–Light Laboratories, Colnbrook, Bucks., U.K. (90–100%) or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. (type VI). Water was redistilled from alkaline permanganate. Methanol (Burroughs Wellcome, London S.E.1, U.K.) was specially purified (Baxendale & Mellows, 1961). Other chemicals were the purest available commercially. Neutral solutions in the absence of buffer were made up under Ar bubbling by dissolving cytochrome *c* in water that had been boiled to remove CO₂, the pH being adjusted to 7.0 ± 0.2 with 0.05 M-H₂SO₄.

Pulse-radiolysis experiments were conducted as in earlier studies (Land & Swallow, 1971, 1974) by using single pulses (less than 1 μ s duration) of 9–12 MeV electrons from a Vickers linear accelerator. Solutions were de-aerated by bubbling with Ar or saturated with N₂O, and were contained in cells of optical path-length 1 mm. Fresh solution was used for each pulse. Changes in absorption caused by the pulses were recorded as a function of time at selected wavelengths by photoelectric detection. Oscilloscope traces showing changes in transmission against time were recorded on polaroid film. The high-radiance Bausch and Lomb monochromator used previously (Land & Swallow, 1971, 1974) (minimum band-

width 2.5 nm) was replaced by a 0.25 m Jarrell Ash monochromator, which allowed resolution down to 0.4 nm. This was calibrated by using suitable lines of a Thermal Syndicate mercury arc lamp. Unless otherwise stated all pulse-radiolysis experiments were at ambient temperature (18–22°C). Absorption spectra of stable solutions were measured with a Unicam SP.8000 spectrophotometer or a Cecil digital spectrophotometer, by using cells of optical path-length 1 cm.

Results

De-aerated aqueous solutions (pH 7.0) of cytochrome *c* (0.1 mM) containing 2 M-2-methylpropan-2-ol but no buffer were given radiation pulses (approx. 3000 rad). In these solutions the hydroxyl radicals produced from the water react with 2-methylpropan-2-ol, leaving hydrated electrons to reduce cytochrome *c*. Additional reduction, amounting to about 10% of that due to hydrated electrons, is caused by hydrogen atoms (Lichtin *et al.*, 1974). An initial increase in absorbance, attributable to the reduction, was seen in the region 410–420 nm. The increase, ΔE_0 , was maximal at 417 nm. After the reduction, the absorbance decreased with a half-life of about 0.1 s, reaching a value that was constant for seconds at least. The value of $\Delta E_0/\Delta E_\infty$ was 1.43, where ΔE_∞ is the difference between the final absorbance of the partly reduced solution and that of the original fully oxidized solution.

Comparison was made between the E_{695} of unbuffered 0.4 mM-cytochrome *c* solutions, pH 7, in the presence and absence of 2 M-2-methylpropan-2-ol. After allowing for the contribution of lower wavelength absorption bands as done previously (Kaminsky *et al.*, 1973), it was found that that part of the absorbance due to the 695 nm band as such was 25% less in the presence than in the absence of 2 M-2-methylpropan-2-ol.

Both the value of ΔE_∞ produced by a 3000 rad pulse and the half-life for the decrease in absorbance

Table 1. Effect of 2-methylpropan-2-ol and phosphate on ratio of initial change to final change in absorbance at 417 nm produced by pulsing 0.1 mM-cytochrome *c*

For details, see the text.

2-Methylpropan-2-ol concentration (M)	Phosphate concentration (mM)	$\Delta E_0/\Delta E_\infty$
2.0	0	1.43
1.5	0	1.12
1.0	0	1.11
0.5	0	1.05
0.1	0	1.01
2.0	0.1	1.40
2.0	1	1.15
2.0	10	1.12

were independent of 2-methylpropan-2-ol concentration in the range 0.1–2M. However, the ratio $\Delta E_0/\Delta E_\infty$ depended on concentration as shown in Table 1. Addition of salt (sodium/potassium phosphate buffer, pH 7.0) to solutions containing 2M-2-methylpropan-2-ol produced no change in ΔE_∞ or half-life, but decreased $\Delta E_0/\Delta E_\infty$ as shown in Table 1. The part of the absorbance of the solutions due to the 695 nm band as such showed a corresponding increase on addition of salt.

When propan-2-ol, ethanol, methanol or ethylene glycol was used instead of 2-methylpropan-2-ol in the 2M buffer-free solutions, the value of $\Delta E_0/\Delta E_\infty$ produced by a 3000 rad pulse was greater than unity in each case, whereas the half-life for the decrease in absorbance was always about 0.1 s. The value of ΔE_∞ attained was in the order propan-2-ol > ethanol > methanol > 2-methylpropan-2-ol > ethylene glycol.

Pulses (5000 rad) were given to N₂O-saturated neutral solutions of cytochrome *c* (0.1 mM) containing 0.1M-sodium formate and 10mM-sodium/potassium phosphate buffer. In these solutions the hydrated electrons react with N₂O giving hydroxyl radicals; hydroxyl radicals and hydrogen atoms then both react with formate, yielding CO₂^{•-} as the sole reducing species. At room temperature ΔE_0 was almost equal to ΔE_∞ , in agreement with previous results for neutral solutions (Land & Swallow, 1971, 1974). However, as the temperature was raised, $\Delta E_0/\Delta E_\infty$ gradually increased, reaching a ratio of about 1.14 at about 60°C, whereas ΔE_∞ remained almost constant. The half-life for the decrease in absorbance became smaller as the temperature increased, being only a few milliseconds at 60°C. Measurements of the E_{695} of neutral solutions of cytochrome *c* (0.5mM) containing 0.1M-sodium formate and 10mM-sodium/potassium phosphate buffer showed that, after allowing for underlying absorptions, the part of the absorbance due to the 695 nm band as such was 75% less at 60°C than at 18°C.

Discussion

The changes taking place in the present systems can be interpreted in terms of Scheme 1, which has been developed from those adopted for alkaline solutions (Lambeth *et al.*, 1973; Land & Swallow, 1974). It is assumed that one form of ferricytochrome *c* is the native form A which has protein conformation P₁ and in which the sulphur atom of methionine-80 is co-ordinated to iron. This form possesses an absorption band at 695 nm. Ferricytochrome *c* can also exist in the form (or forms) B in which changes in protein conformation from P₁ to P₂ have taken place and the sulphur atom has been replaced by another group, X. This form does not have an absorption band at 695 nm.

In neutral solution, the ratio of the concentrations of forms A and B is governed by the concentrations of alcohol and salt, and temperature, and can be estimated from the E_{695} . The radiolytically generated reducing agents (e⁻_{aq}, etc.) are assumed to be able to reduce both forms A and B, giving, in the first instance, forms C and D respectively. Form C differs significantly from form A in oxidation state, but it has the same protein conformation P₁. Similarly, form D differs from form B only in oxidation state. The protein conformation of ferrocyclochrome *c* in solution is thought to be different from that of ferricytochrome *c*, so that, after form C has been formed from form A, its conformation changes to P₃, giving form E. It is proposed that this change does not involve the haem group or its immediate environment, and is not accompanied by any spectroscopic change in the region under consideration. Since ferrocyclochrome *c* is generally considered to be more stable than ferricytochrome *c*, it is assumed that after form D has been formed in solution it also changes to form E. This requires X to be replaced by S and the protein conformation to change from P₂ to P₃.

The hypothesis can be applied to the solutions containing 2M-2-methylpropan-2-ol but no buffer, in which the principal reducing agent is the hydrated electron. Since hydrated electrons are powerful and unspecific reducing agents, it may be assumed that they reduce forms A and B with approximately equal rate constants. ΔE_0 is then given by:

$$\Delta E_0 = \{(\epsilon_C - \epsilon_A)[A] + (\epsilon_D - \epsilon_B)(100 - [A])\} [e^-_{aq}] d / 100$$

where ϵ_C , ϵ_A , ϵ_D and ϵ_B are molar extinction coefficients at 417 nm of forms C, A, D and B respectively, [A] is the percentage of ferricytochrome *c* present in the original solution in the form A, $[e^-_{aq}]$ is the concentration of hydrated electrons produced by the pulse, and *d* is optical path-length. ΔE_∞ is given by:

$$\Delta E_\infty = \{(\epsilon_E - \epsilon_A)[A] + (\epsilon_E - \epsilon_B)(100 - [A])\} [e^-_{aq}] d / 100$$

Scheme 1. Different forms of cytochrome *c*

Dividing these two equations, and putting ϵ_C equal to ϵ_E , we obtain:

$$\Delta E_0/\Delta E_\infty = \frac{(\epsilon_A - \epsilon_A)[A] + (\epsilon_D - \epsilon_B)(100 - [A])}{(\epsilon_E - \epsilon_A)[A] + (\epsilon_E - \epsilon_B)(100 - [A])}$$

The measurements at 695 nm show that $[A]$ is 75, and $\Delta E_0/\Delta E_\infty$ is 1.43. The extinction coefficients of ferri-cytochrome *c* solutions at the Soret maximum depend on the composition of the solution but at 417 nm are less dependent (Kaminsky & Davison, 1969). For simplicity, we have assumed that ϵ_A and ϵ_B are both 83 500 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ (Margoliash & Frohwirt, 1959). Fitting these values into the equation we find $\epsilon_D = 204\,000$ litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$. However, hydrated electrons may reduce form A more rapidly than form B, in which case ϵ_D would be greater, so our value of the extinction coefficient for form D, where X is the group that replaces sulphur when alcohol is added to a neutral solution of cytochrome *c*, becomes $\epsilon_D \geq 204\,000$ litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$. This is similar to the value of $\geq 173\,000$ litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$ found previously for the form of D which appears transiently in alkaline solutions (Lambeth *et al.*, 1973). A similar value may also be deduced from our own results with alkaline solutions (Land & Swallow, 1974).

The decrease in $\Delta E_0/\Delta E_\infty$ as the 2-methylpropan-2-ol concentration is decreased is attributed to the presence of a higher proportion of native cytochrome *c* at the lower concentrations of alcohol. The decrease

in $\Delta E_0/\Delta E_\infty$ with addition of phosphate is explained as an increase in concentration of native cytochrome *c* with addition of phosphate. These explanations are confirmed by the measurements at 695 nm on solutions that had the same composition as those exposed to radiation pulses.

The essential equality of ΔE_0 and ΔE_∞ in unbuffered neutral solutions containing 0.1 M 2-methylpropan-2-ol makes it difficult to see how earlier workers could have observed slow changes after reduction of such solutions (Pecht & Faraggi, 1972). The earlier result could be explained if, for some reason, perhaps connected with the preparation of the solutions, an abnormally high proportion of the 'denatured' form B had been present. The spectroscopic impurity associated with the very high absorbances in the earlier solutions could be an additional, or alternative, factor.

The effect of other alcohols on $\Delta E_0/\Delta E_\infty$ resembles that produced by 2-methylpropan-2-ol, since all alcohols produce modified forms of cytochrome *c* (Kaminsky & Davison, 1969). The variation in ΔE_∞ is consistent with radicals formed by hydroxyl attack on propan-2-ol, ethanol and methanol being able to reduce cytochrome *c*, at various rates and/or efficiencies, whereas the radicals formed from 2-methylpropan-2-ol do not do so. This agrees with work with ethanol and 2-methylpropan-2-ol (Shafferman & Stein, 1974) but disagrees with work with methanol (Wilting *et al.*, 1975). We have no explanation of the earlier results with methanol. The fact that ΔE_∞ with 2-methylpropan-2-ol is greater than ΔE_∞ with ethylene glycol can be explained by hydroxyl atoms reacting more rapidly with ethylene glycol than with 2-methylpropan-2-ol (Anbar *et al.*, 1975), radicals from ethylene glycol being unable to reduce cytochrome *c*.

The effects seen on raising the temperature show that heat, like addition of alcohols or increase in pH, causes the formation of form B from form A. The fact that 75% removal of 695 nm absorption occurs in going from 20° to 60°C, whereas $\Delta E_0/\Delta E_\infty$ only increases by 14%, suggests that form B generated from form A by heat may not be identical with form B formed by increasing the alcohol concentration or, for that matter, increasing pH. Another factor may be that the rate of reaction of CO_2^- with cytochrome *c* is more dependent on protein conformation than is the rate of reaction of the hydrated electron.

The present results show that the change in structure of ferrocycytochrome *c* from a form with the same protein conformation, P₂, as one of the oxidized forms (represented as form D in Scheme 1) to the form with the conformation P₃, characteristic of the normal reduced form (E), proceeds with the same low rate constant in neutral solutions as had been previously observed in alkaline solutions. It seems most likely that this rate is determined by the rate at

which the protein structure adjusts to the altered state of the central iron atom, the replacement of X by methionine sulphur providing the spectroscopic indication that the change is happening. The conformation, P_1 , of the form of ferricytochrome *c*, which is native in aqueous solutions (form A in Scheme 1), is different from that (P_2) of the form B, which enables the slow change to be demonstrated. Consequently, when native cytochrome *c* is reduced, the change from P_1 to P_3 could be much more rapid than that from P_2 to P_3 . However, it is difficult to see why this should be so, since the rate would still be determined by the rate at which the protein structure adjusts to the state of the iron atom. In biological systems the turnover between oxidized and reduced cytochrome *c* is much more rapid than the conformational change observed in this work. It therefore seems likely that the protein conformations of ferricytochrome *c* and ferrocyanochrome *c* in the hydrophobic environment of the biological system are much more alike than they are in aqueous solutions.

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