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EFFECTS OF ALCOHOL/WATER MIXTURES ON THE STRUCTURE AND REACTIVITY OF CYTOCHROME *c*

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Summary

The oxidation reaction of ferrocytochrome *c* (produced in situ by pulse radiolysis) by $Fe(CN)_6^{3-}$, was used to probe the effect of alcohol/water mixtures on the reactivity of the protein. Reduced cytochrome *c* is oxidized in a biphasic process. The relative contribution of each phase depended on: pH, alcohol concentration and temperature. pK_a values were derived from the kinetic data. These pK_a values were identical with the spectroscopic pK_a values determined under similar conditions by monitoring the 695 nm absorption band of the oxidized protein. The two phases of oxidation were therefore related to the oxidation of a relaxed and a nonrelaxed conformer of reduced cytochrome *c* produced in situ. A shift in the pK_a of ferricytochrome *c* and a retardation of the redox reactions of both the reduced and the oxidized protein were observed at low alcohol concentrations (up to 5 mol %). These low alcohol concentrations are known to affect the structure of water (Yaacobi, M. and Ben-Naim, A. (1973) J. Sol. Chem. 5, 425—443; Ben-Naim, A. (1967) J. Phys. Chem. 71, 4002—4007 and Ben-Naim, A. and Baer, S. (1964) Trans. Faraday Soc. 60, 1736—1741) but have only minor effects on the protein. Accordingly, the kinetic results are interpreted on the basis of involvement of water molecules in the reaction complex of cytochrome *c* with its redox substrates.

Introduction

Alcohols affect both structure and reactivity of cytochrome *c* [1]. The reactions of cytochrome *c* with O_2 [2], $Fe[(CN)_5NH_3]^{2-}$ [3], and the isopropyl radical [4] are affected by the presence of alcohols. Alcohols affect also the visible absorption spectrum [5], circular dichroism behaviour [6], complexation with carbon monoxide [7] and the near infrared absorption spection of cytochrome *c* [8]. All these effects were attributed to the opening of the heme

crevice, and the disruption of the sixth coordination bond of Fe with the sulfur of methionine 80.

The effect of alcohols on cytochrome *c* can also be interpreted in terms of their influence on the water-protein structure [9,10].

In this work we report our results on the effect of ethanol on the oxidation of ferrocytochrome *c* (CII), by ferricyanide, and its cumulative effect with pH and temperature, on structure and spectral properties of cytochrome *c*.

Experimental

Materials. Sigma type III horse-heart ferricytochrome *c* (CIII) was used without further purification. It was fully oxidized when $Fe(CN)_6^{3-}$ was present. The concentration of CIII was determined by the 528 nm absorption peak, using $\epsilon = 11.2 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ [11]. Water used was triply distilled. Argon was supplied by Matheson, and was freed of oxygen by bubbling through a solution containing V(II) prepared by in situ reduction of Fluka purum grade $NaVO_3$ with zinc amalgame prepared from B.D.H. analytical reagent zinc and Frutarom analytical grade mercury. *t*-Butanol (Merck, pro-analisi) and ethanol (Fluka for ultraviolet spectroscopy) contained no impurities detectable by ultraviolet absorption spectrometry. $K_3Fe(CN)_6$ was supplied by B.D.H.; $NaH_2PO_4 \cdot H_2O$ by Mallinckrodt; $Na_2HPO_4 \cdot 7H_2O$ by J.T. Baker, and NaOH by Riedle De Haen. All reagents were of analytical grade and were used without further purification.

Apparatus. The Varian linear accelerator of the Hebrew University and the optical and electronic systems were described elsewhere [12]. The irradiation cells were rectangular flow-through cells fabricated of spectrosil. Their optical path was 2 cm. For temperature-effect studies we used a rectangular cell of 3 cm optical path, surrounded by a bigger cell through which water from a thermostatted bath was circulated. Three optical windows of the cell were left free of the thermostatted water for entry of the electron beam and for passing the analytical light through the cell. The temperature within the cell was calibrated using a thermocouple and was found to be equal to that of the water bath within $\pm 0.5°C$.

Spectra of solutions were taken on a Cary 14 spectrophotometer, and pH measurements on a digital pHM52 of Radiometer.

Procedures. The pH of the solutions was adjusted by adding phosphate buffer (in a concentration appropriate for the desired ionic strength) for pH <8, or by adding Na_2HPO_4 and NaOH or H_2SO_4, above pH 8. In the presence of ethanol, the observed pH was higher than in its abosence. In the presence of 0.8 M and 2.5 M ethanol, the recorded pH was higher by 0.1 and 0.25 pH units respectively, as compared with a similar solution containing no ethanol. These increments were independent of pH through the pH range of this work. The pH values cited are corrected values, namely the observed values minus the relevant increment.

Solutions were prepared in glass bottles no more than 15 h before use. Deaeration of solutions was accomplished by sweeping with argon for at least 15 min. Solutions were saturated with gas and irradiated in large glass syringes equipped with capillary standard taper joints. Irradiations were carried out no more than 0.5 h after sweeping with gas.

The absorbed dose per pulse was determined routinely using the spectrum of hydrated electrons produced by pulsing $1 \cdot 10^{-2}$ M aqueous ethanol at pH 9.5—10, taking $\epsilon_{578} = 1.06 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ and $G(e^-_{aq}) = 2.75$ molecules/100 eV [13]. Doses used ranged between 700 and 1500 rads per pulse (equivalent to approx. $2—4 \cdot 10^{-6}$ M of e^-_{aq}). To assure pseudo first order conditions, the concentration of CII produced was approx. 10 times lower than that of $Fe(CN)_6^{3-}$.

The temperature of solutions was 22 ± 1°C, when not studying temperature effect. The kinetics were followed by recording the change in absorption at 550 nm. Spectroscopic titrations of the 695 nm peak were carried out in a cell of 10.0 cm light path. $\Delta A_{695\,nm}$ was calculated by the method of Kaminsky et al. [8].

Oscilloscope traces were analyzed by transferring the data to punched cards by means of a magnifying manual trace follower coupled to an analogue to digital converter and processing the cards in a Control Data Corporation Cyber digital computer.

Results

In order to study the oxidation of CII by $Fe(CN)_6^{3-}$, we produced CII in situ, in the presence of $Fe(CN)_6^{3-}$ according to the reactions presented in Table I, using appropriate concentrations of reagents. In order to prevent reactions with O_2, solutions were freed of O_2 by saturation with argon.

More than 99% of the OH radicals were scavenged by *t*-butanol or by ethanol, according to reactions 5 and 6 (Table I). The *t*-butanol concentration was 0.2 M, approx. 10^4 times larger than that of CIII. *t*-Butanol radicals do not react with C(III) [14,20]. Ethanol concentrations were at least $2.5 \cdot 10^3$ times larger than those of CIII. The concentrations of CIII and of $Fe(CN)_6^{3-}$ were $2—4 \cdot 10^{-5}$ M, and the ratio $[CIII]/[Fe(CN)_6^{3-}]$ was always between 1 and 2. Thus, ethanol radicals were oxidized mainly by $Fe(CN)_6^{3-}$ and not by CIII, and hydrated electrons (e^-_{aq}) reacted mainly with CIII.

Approximately 5 μs after a pulse, reactions 1—9 are practically completed and CII is produced in a yield of approx. 70% of e^-_{aq} [12,14]. At this stage an increase in absorption is observed at 550 nm. After 100 μs, or later, a decrease

TABLE I

REACTIONS OCCURRING IN A PULSE-IRRADIATED SOLUTION CONTAINING CIII, $Fe(CN)_6^{3-}$ AND ALCOHOL

Reaction	$k(M^{-1} \cdot s^{-1})$	Reference
(1) $e^-_{aq} + CIII \rightarrow CII$	$2 \cdot 10^{10}$	14
(2) $e^-_{aq} + Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-}$	$1—2 \cdot 10^9$	15
(3) $H^{\cdot} + CIII \rightarrow CII + H^+$	$1.1 \cdot 10^{10}$	14
(4) $H^{\cdot} + Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-} + H^+$	$5.6 \cdot 10^8$	16
(5) $OH^{\cdot} + (CH_3)_3COH \rightarrow H_2O + \dot{C}H_2C(CH_3)_2OH$	$5 \cdot 10^8$	17
(6) $OH^{\cdot} + CH_3CH_2OH \rightarrow H_2O + CH_3\dot{C}HOH$	$1.8 \cdot 10^9$	17
(7) $OH^{\cdot}$ CIII → products	$1.4 \cdot 10^{10}$	18
(8) $CH_3\dot{C}HOH + Fe(CN)_6^{3-} \rightarrow Fe(CN)_6^{4-} + CH_3CHO$	$5.3 \cdot 10^9$	19
(9) $CH_3\dot{C}HOH + CIII \rightarrow CII + CH_3CHO$	$1.8 \cdot 10^8$	20

in absorption is observed (Fig. 1a), which corresponds to the reoxidation of CII by $Fe(CN)_6^{3-}$:

(Reaction 10) $CII + Fe(CN)_6^{3-} \rightarrow CIII + Fe(CN)_6^{4-}$

Results obtained in the presence of 0.1 M ethanol, or 0.2 M *t*-butanol (Table II), are similar to results obtained previously without alcohol (ref. 21 and references therein). At neutral pH, in the presence of salts such as sulphate and phosphate, the reaction is monophasic, and second order. When higher concentrations of alcohol are used, two phases of oxidation are observed at neutral pH. Again, each phase is a second order process, first order in CII, and in $Fe(CN)_6^{3-}$ (Fig. 1, b and c).

Two phases of oxidation were also observed at lower concentrations of alcohols (up to 0.2 M), or in their absence [21], but at a higher pH range. Both phases were second order, but rates are somewhat faster than those at higher alcohol concentrations (Table II).

A plot of the relative amount of CIII produced by each phase of oxidation as a function of pH, gives a titration curve from which a pK_a value can be derived. Two such titrations, carried out in two concentrations of ethanol are shown in Figs. 2 and 3. The pK_a values derived from these titrations are summarized in Table III, together with the pK_a value derived in the presence of a very low concentration of alcohol. Figs. 2 and 3 also show the titration curves of the 695 nm absorption peak of CIII in similar concentrations of ethanol. The spectroscopic pK_a values obtained are summarized in Table III. The similarity between the values derived from the kinetic titrations and from the spectroscopic titrations is plausible.

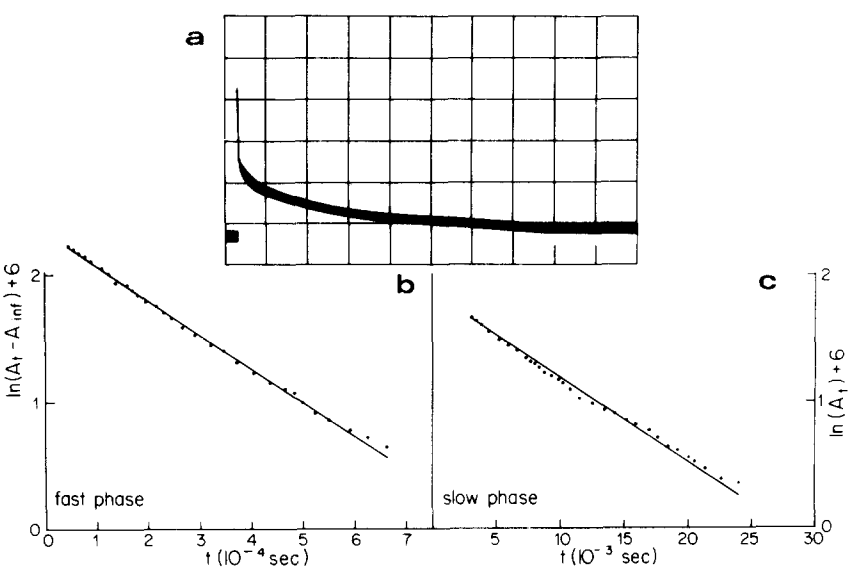

Fig. 1. Two phases of oxidation of CII (produced in situ), by $Fe(CN)_6^{3-}$, in the presence of 2.5 M ethanol. pH = 8.5; μ = 0.1; [CIII] = $2 \cdot 10^{-5}$ M; [$K_3Fe(CN)_6$)] = $2 \cdot 10^{-5}$ M. a. Oscilloscope trace. Abscissa: 5 ms per division; ordinate: 10 mV per division (photomultiplier output). V_0 = 400 mV. b. Pseudo first order plot of the faster phase. c. Pseudo first order plot of the slower phase.

TABLE II

THE EFFECT OF ALCOHOL [a] CONCENTRATION ON THE RATE CONSTANTS OF THE SLOW AND FAST PHASES OF OXIDATION

Solutions contained $2–4 \cdot 10^{-5}$ M CIII, $2–4 \cdot 10^{-5}$ M $K_3Fe(CN)_6$. Ionic strength achieved with phosphate (pH 8.5). All values of rate constants are a mean value of at least 5 independent measurements, and are accurate within ± 10%.

Alcohol	Concentration	μ	k_{slow} ($M^{-1} \cdot s^{-1}$)	k_{fast} ($M^{-1} \cdot s^{-1}$)	L [b] slow	L [b] fast
mol %	M					
0.18	0.1	0.1	$7.8 \cdot 10^6$	$2.9 \cdot 10^8$	1.0	1.0
0.36 [c]	0.2 [c]	0.1	$8.2 \cdot 10^6$	$3.0 \cdot 10^8$	0.95	0.97
1.5	0.8	0.1	$5.5 \cdot 10^6$	$2.6 \cdot 10^8$	1.4	1.1
3.2	1.6	0.1	$4.5 \cdot 10^6$	$1.6 \cdot 10^8$	1.7	1.8
5.0	2.5	0.1	$3.2 \cdot 10^6$	$1.3 \cdot 10^8$	2.4	2.2
0.18	0.1	0.02	$3.0 \cdot 10^7$	$8.3 \cdot 10^8$	1.0	1.0
5.0	2.5	0.02	$1.0 \cdot 10^7$	$4.5 \cdot 10^8$	3.0	1.8
0.18	0.1	0.005	$1.4 \cdot 10^8$	$1.8 \cdot 10^9$	1.0	1.0
5.0	2.5	0.005	$1.0 \cdot 10^8$	$1.2 \cdot 10^9$	1.4	1.5

[a] Ethanol, unless otherwise indicated.

[b] L is the ratio between the rate constants in the presence of 0.1 M ethanol and in the presence of the stated concentration of alcohol.

[c] Alcohol present, t-butanol.

Parallel experiments in the presence and absence of alcohol were also carried out at temperatures from 5 to 35°C. No effect of temperature on the kinetics of CII oxidation by $Fe(CN)_6^{3-}$ was observed. However, the relative amount of each phase of oxidation was dependent on temperature, the faster phase

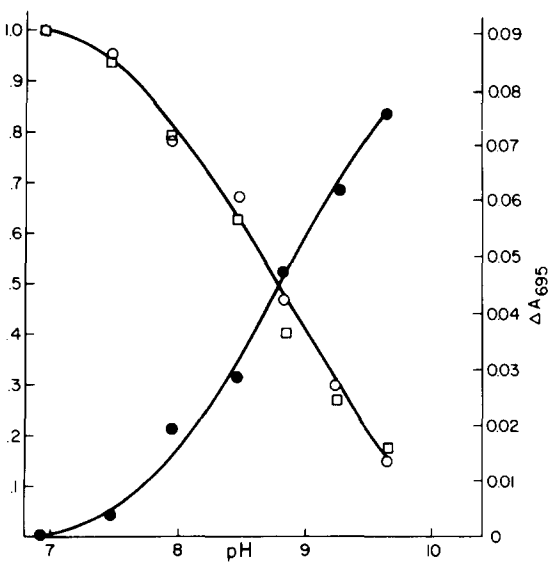

Fig. 2. Kinetic and spectroscopic titration curves in the presence of 0.8 M ethanol. Kinetic titration curve (left scale): relative amount of CIII produced by each phase of oxidation versus pH. •, Fast phase; ○, slow phase. Spectroscopic titration curve (right scale): ΔA_{695nm} versus pH (□) [CIII] = $4 \cdot 10^{-5}$ M; [$K_3Fe(CN)_6$] = $2 \cdot 10^{-5}$; μ = 0.1. The indicated pH values are the corrected values (see Procedures).

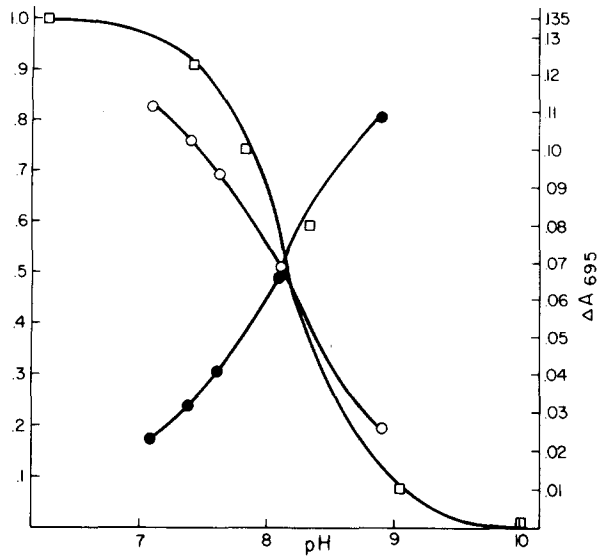

Fig. 3. Kinetic and spectroscopic titration curves in the presence of 2.5 M ethanol. Details as in legend to Fig. 2.

TABLE III

pK_a VALUES DERIVED FROM THE TITRATION CURVES SHOWN IN FIGS. 2 AND 3

Alcohol [a]	Concentration	Kinetic titration pK_a	Optical titration pK_a
mol %	M		
0.36 [b]	0.2 [b]	9.2 [c]	—
1.6	0.8	8.9	8.9
5.0	2.5	8.1	8.2

[a] Ethanol, unless otherwise indicated.
[b] Alcohol used, *t*-butanol.
[c] Value from ref. 21.

TABLE IV

CALCULATED pK_a VALUES (ACCORDING TO EQN. 1)

μ = 0.1 phosphate.

t(°C)	Ethanol (0.1 M)	Ethanol (2.5 M)
7	9.2	8.3
22	9.2	8.1
33	8.8	7.8

becoming larger at higher temperatures both in the presence and in the absence of alcohol.

pK_a Values, at three temperatures and two ethanol concentrations were calculated (Table IV). These pK_a values were derived using Eq. 1:

$$pK = pH + \log \frac{A(\text{slow phase})}{A(\text{fast phase})} = \text{pH} + \log \frac{[\text{neutral form}]}{[\text{basic form}]}$$

where A is the difference in absorption measured at zero and infinity time in each phase of oxidation.

Discussion

The observed effects of alcohols on the physical properties of cytochrome c are rather small at concentrations lower than 5—10 mol %, and become very pronounced at concentrations exceeding these values [5—8]. Ethanol does not affect the Soret band [5], and the circular dichroism spectrum of CIII [6], up to a concentration of approx. 10 mol %. The observed effects were suggested to originate in a conformational change in the protein, which exposes the heme crevice. Alcohol concentrations exceeding 10 mol % may produce such a distortion in the native configuration of the protein, that conclusions on relationship between structure and function may be meaningless. Therefore, in the present study, concentrations of alcohols not exceeding 5 mol % were used.

As a dynamic probe for the effect of alcohols on the structure and function of cytochrome *c* we used the well characterized reaction between CII and $Fe(CN)_6^{3-}$ [21].

In the presence of alcohol, two phases of oxidation are observed. The relative contribution of each phase depends on the pH. The interpretation of this phenomenon is based on the presence of two conformers of CII produced in situ: One form of CII is a relaxed form produced from the neutral isomer of CIII (which has a pK of 9.3 in the absence of alcohols [1]). The other is a non-relaxed form, produced from the basic isomer of CIII, having a more open conformation than the relaxed form. This interpretation is consistent with the observations of slow intramolecular changes in pulse-radiolytically produced CII at basic pH [22,23].

The spectroscopic titrations shown in Figs. 2 and 3 give the pK_a of the alkaline isomerization of CIII in the presence of alcohol (Table III). The similarity between the kinetic and spectroscopic pK_a values indicates that indeed the oxidation of CII by $Fe(CN)_6^{3-}$ titrates the different isomers of CIII. This interpretation is consistent with the recent observation of Land and Swallow [24], of an unstable conformer of CII produced on reduction of CIII at neutral pH, in the presence of alcohol.

The alkaline isomerization of CIII is considered to be composed of an acid-base equilibrium, with $pK_a \approx 11$, and a conformation change with an equilibrium constant of approx. 125. The overall pK of this isomerization is approx. 9 [25]. The $pK_a \approx 11$ of the acid-base equilibrium was correlated with an equilibrium of a lysine, which coordinates to the iron in the basic form of CIII [1,25].

Most of the redox reactions of cytochrome *c* with small reagents are thought

to take place by a remote attack near the exposed edge of the heme [1]. $Fe(CN)_6^{3-}$ was shown to form an electrostatic complex with the protein [21, 26,27,28], and it was suggested there that the site of interaction is a cluster of lysines near the exposed edge of the heme [1]. The interaction of this cluster, with water, which should be affected by alcohols present in the solution, should influence in turn reactions taking place via this site.

Alcohol/water mixtures were shown to behave differently in low and in high alcohol concentrations [29–31]. In very low alcohol concentrations (up to approx. 3 mol %), alcohols stabilize the structure of water. At higher alcohol concentrations (approx. 3–20 mol %) alcohols decrease the structure of water. In the range of up to approx. 10 mol %, the changes in the thermodynamic properties of the mixtures are quite small, usually showing a small extremum around 3 mol % [29–31]. At low alcohol concentrations big changes in cytochrome *c* structure do not occur. Small changes, however, do occur, as manifested by the change of the pK_a of CIII (Table III), and by the retardation of the rates of redox reactions of the protein (Table II and refs. 2–4). These small changes occur mainly around amino acid residues on the surface of the protein, such as hydrophylic lysines, and involve water molecules in contact with these residues.

The effect of perchlorate, a water structure breaking anion, on the oxidation of CII by $Fe(CN)_6^{3-}$ is similar to the effect of alcohols. This effect was also interpreted as manifesting the effect of water structure on the reactions of cytochrome *c* [21]. There is, however, some difference between the effects of alcohol and perchlorate. Though both slow the oxidation of CII by $Fe(CN)_6^{3-}$ and decrease the kinetic pK_a of CIII, alcohols affect its spectroscopic pK_a whereas perchlorate does not. It seems therefore, that alcohols affect the acid-base equilibrium of the lysine that replaces methionine 80 as the heme iron sixth ligand, whereas perchlorate has some other effects.

The oxidation of CII by $Fe(CN)_6^{3-}$ was shown to be affected by changing the solvent from H_2O to D_2O [10]. This is consistent with the involvement of water in some stage of the redox reaction, and with the participation of water molecules in the structure involved in the reaction region. Involvement of water molecules in delicate conformation changes was also suggested by Margalit and Schejter [32].

Temperature and alcohols have a cooperative effect on the pK_a of the isomerization of ferricytochrome *c* (Table IV). A significant change in pK_a takes place when the temperature is changed from 22 to 35°C. Since in mitochondria cytochrome *c* does not exist in a pure aqueous solution, the results shown in Table IV might indicate that in the physiological temperature, CIII exists in mitochondria as a mixture of two conformers. If both conformations are reducible by the physiological reductant of CIII (cytochrome *c* reductase), then both relaxed and nonrelaxed CII are formed. However, the relaxation time of the 'open' conformer is approx. 0.1 s [22–24], which is too long on the time scale of electron transfer in the oxidative chain in mitochondria. If only one conformation is reducible by cytochrome *c* reductase, the concentration of CIII effective in redox activity in mitochondria is lower than its total concentration.

In conclusion, low concentrations of alcohols which do not change dramatically the structure and the physical properties of cytochrome *c*, but produce

changes in the structure of water, cause small changes in the structure of the protein. This is manifested by the shift in the pK_a of CIII, and also in the retardation of the redox reactions of both CIII and CII. The kinetic effect (the retardation) indicates as well that water molecules participate in the reaction complex of cytochrome *c* with its redox substrates.

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