The Reactivity of Conformationally Modified Cytochrome *c*†

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ABSTRACT: Lyophilization of ferricytochrome *c* produces modified low spin forms of the protein which do not have the native methionine-80–heme iron bond. These forms revert to native cytochrome *c* at different rates when the lyophilized sample is redissolved. These modified cytochromes *c* provide a system for the investigation of electron-transfer pathways in cytochrome *c* which eliminates the inherent disadvantages of the frequently used chemical modification approach. At least two of these modified forms are not reducible by succinate–cytochrome *c* reductase. Another form(s) reverts too rapidly for determination of its activity. A conformational change appears to occur with lyophilized ferrocytochrome *c* when it is dissolved but this only slightly affects its activity with cytochrome oxidase. The liganding of methionine-80 to the heme iron in ferricytochrome *c* may thus be essential for its activity with the reductase.

The biological function of cytochrome *c* in the mitochondrion involves its interaction with two respiratory components, cytochrome c_1 and cytochrome oxidase. One of the probes for the mechanisms of electron transport is chemical or enzymic modification of cytochrome *c* (*e.g.*, Margoliash *et al.*, 1973). Many of these modifications reported involve alterations which may manifest secondary effects and complicate interpretations of the results. The recent report of Aviram and Schejter (1972) which demonstrated that lyophilized cytochrome *c* is in a conformationally modified low spin form immediately on being dissolved provides an interesting modification for structure–function studies. In this case Aviram and Schejter (1972) have claimed that the methionine-80–heme iron ligand is displaced by another strong field ligand to produce conformational changes in the absence of chemical modification. Such a modification would be free from the complications associated with chemical or enzymic modification studies and would permit an examination of the role of methionine-80 in cytochrome *c* activities in a more direct manner. Moreover, lyophilization is a relatively mild process in contrast to some drastic reactions in certain chemical or even enzymic modifications. We report here on our studies of the reactivity of this modified cytochrome *c* with succinate–cytochrome *c* reductase and cytochrome oxidase.

Materials and Methods

Cytochrome *c* was obtained from Sigma, Type III. Succinate–cytochrome *c* reductase and cytochrome oxidase were prepared essentially as reported previously (Kuboyama *et al.*, 1972; Yu *et al.*, 1973) and were kindly supplied by Drs. C. A. Yu and L. Yu of this laboratory. Lyophilized ferrocytochrome *c* was prepared by reducing ferricytochrome *c* with dithionite and separated from reagents on a Sephadex G-25 column. The eluate was lyophilized and the product was 87% reduced. Water was double distilled and deionized.

Rates of conformational changes, reduction, and oxidation were determined with a Gilford spectrophotometer, Model 2400, fitted with thermospacers. Low-temperature spectrophotometric measurements were made as described previously (Yu *et al.*, 1972).

In all experiments with freshly dissolved cytochrome *c*, the lyophilized cytochrome *c* was weighed into a test tube and dissolved in the buffer solution by vortexing for 10 sec. The solution was then immediately transferred to the cuvet in the spectrophotometer. Reduction and oxidation were monitored by observing the increase or decrease in cytochrome *c* absorbance at 550 nm. The change of absorbance at 695 nm was used as a measure of the change of conformation and spin state (Schejter and George, 1964).

First-order rate constants were determined from plots of $\ln (A_\infty - A_t)$ *vs.* time. For polyphasic reactions rate constants were computed by extrapolation of the slower phase back to zero time and subtraction of the extrapolated values from those of the faster step. All rate constants reported are the average values obtained from five experiments with standard deviations listed in the table.

Results and Discussion

The rates of conformation change of lyophilized ferricytochrome *c* immediately after solution were determined at 3°; the low temperature was used in an effort to decrease the rate of change and thereby permit reactivity studies on the modified form. At this temperature the conformation change was biphasic and yielded two first-order rate constants (*cf.* Table I). Aviram and Schejter reported only a monophasic conformation change at room temperature (Aviram and Schejter, 1972). At 3° the 695-nm absorbance band of the lyophilized ferricytochrome *c* had, however, already developed to 50% of its value in the native state during the 10 sec required to dissolve the lyophilized sample and place it into the spectrophotometer. When the sample was frozen in liquid nitrogen immediately after dissolving (3 sec) in buffer at 3° and the spectrum determined at −196°, the 695-nm band, as shown in Figure 1, had only

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TABLE I: First-Order Rate Constants for Conformational Changes, Reduction, and Oxidation of Freshly Dissolved and Native Cytochrome *c*.[a]

State of Cytochrome *c*	Temp (°C)	First-Order Rate Constant		
		Reduction ($sec^{-1} \times 10^{-2}$)	Oxidation ($sec^{-1} \times 10^{-2}$)	Conformational Change ($sec^{-1} \times 10^{-2}$)
Freshly dissolved	23	1.69 ± 0.11	4.56 ± 0.17	1.6[b]
Native	23	1.84 ± 0.05	5.02 ± 0.26	
Freshly dissolved	3	1.56 ± 0.18	2.37 ± 0.20	
		0.74 ± 0.22		0.79 ± 0.17
		0.04 ± 0.01		0.05 ± 0.01
Native	3	1.57 ± 0.05	1.95 ± 0.03	

[a] The assay for the enzymic reduction of cytochrome *c* was performed in a system containing 0.1 M phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.02 M sodium succinate, 0.2 mg/ml of ferricytochrome *c*, and 10 μg/ml of succinate–cytochrome *c* reductase. The assay for the enzymic oxidation of cytochrome *c* was performed in a system containing 0.1 M phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.2 mg/ml of ferrocytochrome *c*, and 90 μg/ml of cytochrome oxidase for assays at 3° or 45 μg/ml for assays at 23°. Conformation changes were determined on 6.4 mg/ml of ferricytochrome *c* in 0.1 M phosphate buffer (pH 7.4) as changes of absorbance at 695 nm. [b] The value, 1.6, is from Aviram and Schejter (1972).

developed to about 18% of its final value. This value was increased by further delay in freezing the solution. There is thus apparently an initial very rapid conformational change on dissolving lyophilized ferricytochrome *c* followed by the observed intermediate and slow phases.

The spectrum of lyophilized ferricytochrome *c*, determined in the solid state on a thin film by reflectance spectroscopy, had neither a 695- nor 620-nm absorbance band. The latter indicates the absence of any high-spin component in the lyophilized sample (see also Brill and Williams, 1961). This observation is in agreement with the results from Raman spectrophotometry recently reported by Loehr and Loehr (1973). The absence of the 695-nm band indicates that methionine-80 is not liganded to the heme iron as in native cytochrome *c* (Schechter and Saludjian, 1967; Sreenathan and Taylor, 1971). All these results suggest that lyophilized ferricytochrome *c* exists in at least three different low spin forms, none of which has methionine-80 as a heme iron ligand, and which revert to the native state at widely varying rates. Our kinetic data do not exclude the possibility of a three-step reversion of a single modified form of cytochrome *c* but experiments with the reductase, to be discussed later, may favor the former interpretation.[1]

When freshly dissolved ferricytochrome *c* was reduced with soluble succinate–cytochrome *c* reductase at 3° the reaction was computed to be triphasic yielding three first-order rate constants (see Table I and Figure 2) with correlation coefficients exceeding 0.998. Attempts to produce a breakdown of the data into two linear plots yielded significantly lower correlation coefficients.[1] The rate of the initial step was identical with that obtained for the reductase-catalyzed reduction of native cytochrome *c*. The intermediate and slow reduction steps had rate constants closely resembling those for the conformational change of lyophilized cytochrome *c* on being dissolved (Table I).

We interpret these results to indicate that the two forms of conformationally modified cytochrome *c* which revert to

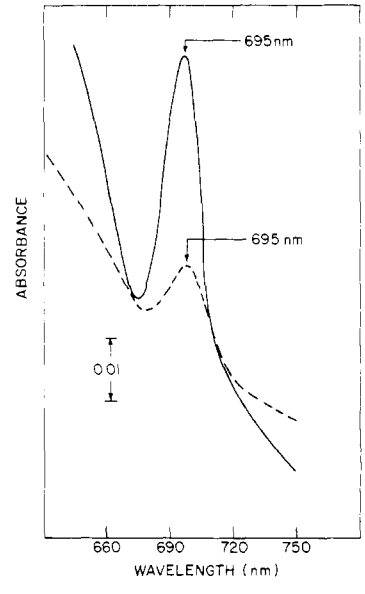

FIGURE 1: Absorption spectra of cytochrome *c*: (—) "native" ferricytochrome *c*; (- - -) freshly dissolved lyophilized ferricytochrome *c*. Both solutions were 8 mg/ml of 50 mM phosphate buffer (pH 7.4); optical path was about 2 mm and temperature, −196°.

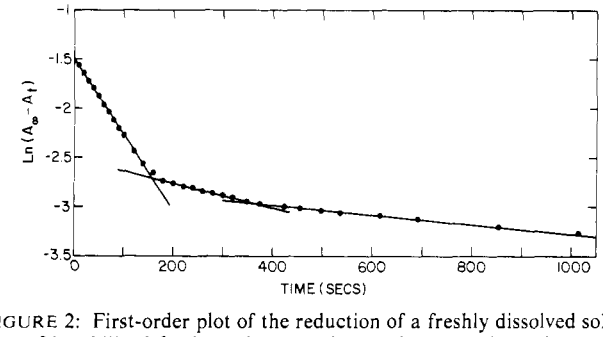

FIGURE 2: First-order plot of the reduction of a freshly dissolved solution of lyophilized ferricytochrome *c* by succinate catalyzed by succinate–cytochrome *c* reductase at 3°. The system contained cytochrome *c*, 0.2 mg/ml; succinate, 0.02 M; and succinate–cytochrome *c* reductase, 10 μg/ml; in 0.1 M phosphate buffer (pH 7.4). The reaction was monitored at 550 nm. The three linear plots were computed with a correlation coefficient of 0.998 or greater.

[1] The number of the intermediate forms as well as whether the reaction proceeds sequentially or otherwise is not the central theme of the paper and does not affect our conclusion about the essentiality of the linkage between the heme iron and methionine-80 in cytochrome *c* for its electron-transfer capability.

the native state more slowly are not reducible by the reductase. Their rates of reduction are controlled by their rates of conformational change which explains the similarity of the respective rate constants. The rapidly changing form of the lyophilized cytochrome *c* had already reverted at the commencement of the reduction and thus the initial rate observed is identical with that obtained with native cytochrome *c*. No information concerning the reactivity of this modified state of cytochrome *c* with the reductase can thus be obtained.

The slightly reduced rate constant for the reductase-catalyzed reduction of freshly dissolved cytochrome *c* at room temperature (Table I) is probably a composite of these reactions which could not be resolved at room temperature.

The three low-spin states[1] of lyophilized cytochrome *c* we have observed could arise from the removal of water leading to conformational changes which induce different residues (*e.g.*, lysine-79, -86, or -13) (Dickerson *et al.*, 1971) to ligand to the iron. An alternative explanation is that these forms have the same ligand but different protein conformations which revert to the native state at differing rates.

No spectral change with time was noted when lyophilized ferrocytochrome *c* was dissolved rapidly by vortexing. The slightly enhanced rate of oxidation of immediately dissolved ferrocytochrome *c* relative to native cytochrome *c* by cytochrome oxidase (Table I) implies that the lyophilized ferrocytochrome *c* is modified in some manner but the reversed order of oxidation at room temperature makes interpretation of these results difficult (Table I). Since the methionine-80–heme iron bond is far stronger in ferro- than in ferricytochrome *c* (Harbury *et al.*, 1965), however, it cannot automatically be concluded that this bond is also disrupted when ferrocytochrome *c* is lyophilized.

In conclusion, lyophilization of ferricytochrome *c* induces conformational changes resulting most probably in the displacement of methionine-80 from the heme iron and produces at least three different forms[1] of modified cytochrome *c*. The failure of two of these forms to be reduced by succinate-cytochrome *c* reductase suggests the requirement of methionine-80 as a ligand of the heme iron for activity in the reductase system. A similar change could possibly occur with reduced cytochrome *c* which does not appreciably alter its activity in the oxidase system. These results may be in line with the suggestion that there are different pathways for the electron during oxidation or reduction of cytochrome *c* (*e.g.*, Margoliash *et al.*, 1973).

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