THERMODYNAMIC AND BINDING PROPERTIES OF CARBOXYMETHYLATED CYTOCHROME *c*

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1. Introduction

One of the approaches to the study of electron transfer pathways in and out of cytochrome *c* is through chemical modification of individual aminoacids considered important in the electron transport process. Selective modifications of tryptophan 59 [1], tyrosines 67 [2] and 74 [3] and methionines 65 and 80 [4] have been described and the spectral and functional properties of the modified molecules elegantly explored. The conclusion which had been drawn on the basis of these studies was that the pathways for electrons into cytochrome *c* from the reductase side and out of the molecule towards the oxidase are nonidentical [3]. We have shown, however, that one of the modifications of that type, formylation of tryptophan 59, leads to a change in thermodynamic and binding properties of cytochrome *c* [5] which can account for the observed modified reactivity of the molecule. In this paper we report the study on di-methionyl-carboxymethylated cytochrome *c* [di-Met (Cm) cytochrome *c*] in which the methionyl residues at positions 65 and 80 are alkylated.

2. Materials and methods

Cytochrome *c* (Sigma, type VI) was alkylated at methionyl residues 65 and 80 according to the method of Schejter and Aviram [4]. The reaction was terminated by dialysis against 5 mM phosphate buffer pH 7.2 at 4°C. After dialysis the solution was centrifuged at 8000 *g* for 10 min to remove any insoluble material.

Pigeon heart mitochondria were prepared by the method of Chance and Hagihara [6] and rat liver mitochondria according to Schneider [7]. Cytochrome *c* depletion was carried out either by the procedure of Jacobs and Sanadi ([8], rat liver mitochondria) or by a modification of this procedure described by Boveris et al. ([9], pigeon heart mitochondria). Cytochrome *c* oxidase was isolated from pigeon breast mitochondria by the method of Fowler et al. [10]).

2.1. *Oxidation–reduction potential measurements*

Potentiometric titrations were carried out by using simultaneous measurements of absorbance and oxidation–reduction potential in an anaerobic cuvette, in the presence of redox mediators [11]. K-ferricyanide was used as the oxidant and Na-dithionite as the reductant. The redox mediators used were: diaminodurene (Aldrich Chem. Co., Milwaukee, WI), phenazine methosulfate (Sigma Chem. Co., St. Louis, Mo), phenazine ethosulfate (K & K, Plainview, NY), duroquinone (Aldrich), pyocyanine (K & K) and 2-hydroxynaphthoquinone (K & K).

2.2. *Binding of cytochrome* c

Binding of cytochrome *c* to the depleted membranes was carried out as described previously [12]. Millimolar extinction coefficients used for the calculations were 19.7 cm^{-1} for native cytochrome *c* and 11 cm^{-1} for the modified cytochrome *c* at 550–540 nm (reduced–oxidized).

2.3. *Oxygen uptake*

Oxygen uptake was measured at 24°C using a Clark oxygen electrode in 0.20 M sucrose, 0.05 morpholinopropane sulfonate pH 7.2 buffer with succinate as the substrate.

Cytochome *c* oxidase activity was determined by the procedure of Smith and Conrad [13].

Cytochrome *a* concentration (half of the total heme *a* content) was measured at 605–630 nm (reduced–oxidized) using an extinction coefficient of 26 cm^{-1}. Protein was determined by the biuret method [14].

3. Results

3.1. *Spectral properties of di-Met-(Cm)-cytochrome* c

The optical spectrum of ferric di-Met-(Cm)-cytochrome *c* was strongly pH-dependent. Below pH 3.5, the spectrum showed the presence of a 620 nm absorption band characteristic of high spin heme proteins. Above pH 7.0 only the low spin form was present. The 695 nm absorption band was absent at all pH values. The reduced form of the cytochrome bound carbon monoxide, oxygen and cyanide as manifested by the appearance of the characteristic absorption spectra of these compounds. These spectral properties of the di-Met-(Cm)-cytochrome *c* are essentially identical with those described by Schejter and Aviram [4].

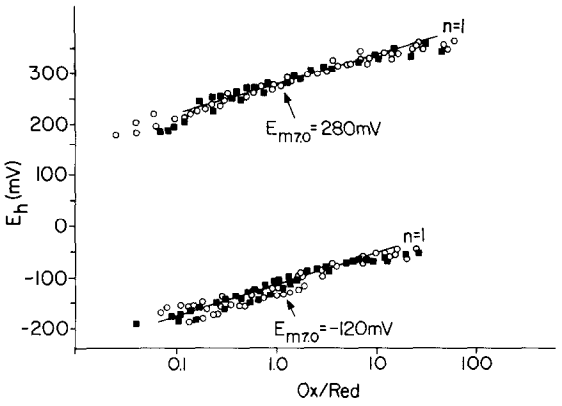

Fig.1. Potentiometric titration curves of native and di-Met-(Cm)-cytochrome *c*. The cytochromes were suspended at a concentration of 6 μM in 0.2 M sucrose, 0.05 M morpholinopropane sulfonate pH 7.0 buffer. The redox mediators used were: 15 μM diaminodurene, 50–100 μM each of phenazine methosulfate and phenazine ethosulfate, 30 μM duroquinone, 9 μM pyocyanine and 15 μ M 2-OH, 1,4-naphthoquinone. The half-reduction potential of di-Met (Cm) cytochrome *c* remained the same when 2-OH-1,4-naphthoquinone concentration was varied by a factor of 5 (5–25 μM) (■) reductive titration; (○) oxidation titration. Temp. 22–25°C.

3.2. *Thermodynamic properties of di-Met-(Cm)-cytochrome* c

Anaerobic potentiometric titrations of the modified cytochrome *c* in sucrose, morpholinopropane sulfonate pH 7.0 buffer yield a straight line with a slope of 60 mV per tenfold change in the ratio of oxidized to reduced cytochrome (fig.1). This indicates that di-Met-(Cm)-cytochrome *c*, similar to its parent molecule, is a one electron donor/acceptor. The half reduction potential at pH 7.0 (E_m 7.0) of the modified cytochrome is – 120 mV ± 10, a value which is nearly 400 mV more negative than that for the native cytochrome *c* under the same pH value and ionic strength of the medium.

3.3. *Binding properties of di-Met-(Cm)-cytochrome* c

In contrast to native cytochrome *c*, its alkylated derivative does not bind easily to depleted mitochondria. Fig.2 compares the binding of normal and di-Met-(Cm)-cytochrome *c* to cytochrome *c*-depleted pigeon heart mitochondrial membranes in the form of a Scatchard plot. The results are calculated relative to cytochrome *a* content in order to gain insight into the stoichiometry of binding. In agreement with our previous studies [6], the binding curves were nonlinear for both *c* cytochromes which indicates heterogeneity in the binding sites. With native cytochrome *c*, high affinity binding sites (K_D = 0.04 μM) are present in the amount of 2 per cytochrome *a* as determined by extrapolation of the steeper portion of the binding curve. Di-Met-(Cm)-cytochrome *c* binds with an affinity approximately 100 times lower (K_D = 1.5 μM) although there also seem to be two high affinity binding sites per cytochrome *a*. Low affinity binding sites are present in larger numbers and the binding constant is approximately 10-fold higher.

3.4. *Reaction of di-Met (Cm) cytochrome* c *with cytochrome* c*-depleted mitochondria*

The di-Met-(Cm)-cytochrome *c* (E_m 7.0 = –120 mV) was not reduced by ascorbate or ascorbate plus *N,N,N*^{1},*N*^{1}-tetramethyl-*p*-phenelenediamine (TMPD) (in contrast to the results of Margoliash et al. [3]) and therefore could not be used as a substrate for cytochrome *c* oxidase. It was also inactive as an acceptor in the reaction with mitochondrial succinate-cytochrome

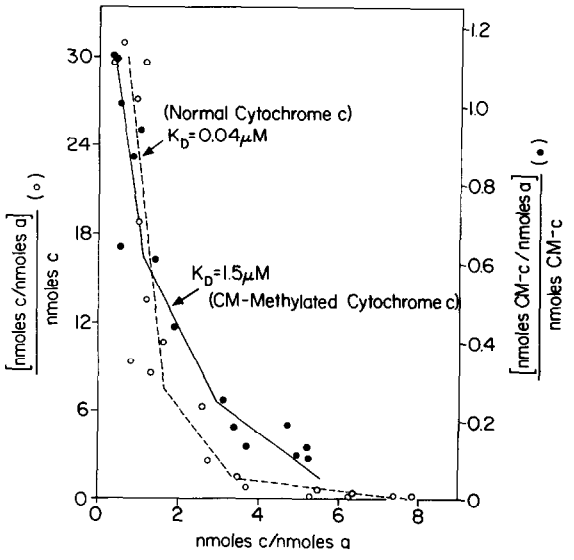

Fig.2. Binding of native and di-Met-(Cm)-cytochrome *c* to cytochrome *c* depleted pigeon heart mitochondria. Cytochrome *c* depleted mitochondria (about 2 μM cytochrome *a*) were incubated in 0.2 M sucrose, 0.05 M morpholinopropane sulfonate pH 7.0 buffer with various amounts of cytochromes *c* for 2 min at room temperature. The suspensions were centrifuged for 10 min at 8000 *g*. The pellet was suspended in 0.1 M phosphate buffer pH 7.2 containing 1% Triton X-100. The concentrations of cytochrome *c* in the pellet and in the supernatant were determined as described in the Methods. (○) horse heart cytochrome *c*, (●) di-Met (Cm) cytochrome *c*. (Values from 3 independent experiments).

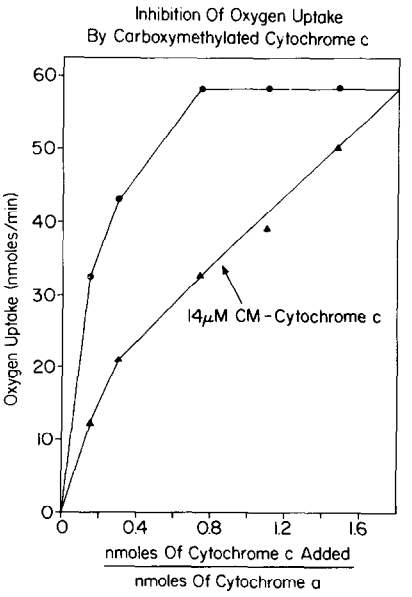

Fig.3. Restoration of oxygen uptake by cytochrome *c* in cytochrome *c*-depleted rat liver mitochondria in the presence and absence of di-Met-(Cm)-cytochrome *c*.
Rat liver mitochondria (0.5 μM cytochrome *a*) were suspended in 0.2 M sucrose, 0.05 M morpholinopropane sulfonate pH 7.0 buffer. The respiratory substrate was 10 mM succinate. Temp. 22°C.

c reductase. In agreement with Schejter and Aviram [4] we found that the modified cytochrome was unable to restore oxygen uptake in cytochrome *c*-depleted mitochondria. Fig.3 shows, however, that when di-Met-(Cm)-cytochrome *c* was added prior to the native cytochrome, higher concentrations of the latter were required to attain the same respiratory activity. Since the *same* final respiratory activity was reached in the presence and absence of di-Met-(Cm)-cytochrome *c* a conclusion which can be drawn is that the modified molecule binds to the same site as the native cytochrome *c* and competitively inhibits oxygen uptake. This suggestion was confirmed by independent experiments in which the activity of isolated cytochrome *c* oxidase (procedure of Fowler et al. [10]) was assayed according to the method of Smith and Conrad [13] in the presence and absence of di-Met-(Cm)-cytochrome *c*.

4. Discussion

Alkylation of the two methionyl residues at positions 65 and 80 in cytochrome *c* results in modification of the properties of this enzyme. Methionine 80 is deeply buried inside the cytochrome *c* and occupies the 6th ligand position whereas methionine 65 is located more superficially. Consequently modification of the former residue leads to changes in the conformational and functional properties of cytochrome *c* while modification of only methionine 65 [15,16] yields a molecule with properties indistinguishable from those of native cytochrome *c*.

The half-reduction potential of di-Met-(Cm)-cytochrome *c* is approximately 400 mV more negative than that of the native enzyme. In mitochondria cytochrome *c* accepts electrons from cytochrome c_1 (E_m 7.0 = 230 mV [17,18]) and passes them to cytochrome *a* [E_m 7.0 = 210 mV (19)]. From the difference between the E_m values of cytochrome *c* (or Di-Met-(Cm)-cytochrome

c) and cytochrome c_1, the value of the equilibrium constant between the two can be calculated. This equals 10^6 in the case of di-Met-(Cm)-cytochrome *c* in contrast to the value of 1 for native cytochrome *c* in mitochondria [20]. Assuming that the rate constant for the reduction of cytochrome c_1 by reduced di-Met-(Cm)-cytochrome *c* is the same as for native cytochrome *c* [21], that for the reduction of the modified molecule by cytochrome c_1 must be 10^6 smaller than the value for native cytochrome *c*. This explains the fact that no reduction of di-Met-(Cm)-cytochrome *c* was observed either by isolated succinate-cytochrome *c* reductase or in mitochondria.

Alkylation of the methionyl 80 residue leads also to conformational changes in the molecule of cytochrome *c* which are manifested by an increase in the binding constant by nearly 100-fold. A similar conclusion was drawn for the other modified *c* cytochromes either on the basis of direct binding studies [12] or circular dichroism spectroscopy [22]. A general conclusion is that as elegant as these studies may be, they tell us little about the electron transfer pathways through the cytochrome *c* molecule.

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