MAPPING OF THE INTERACTION DOMAIN FOR PURIFIED CYTOCHROME c_1 ON CYTOCHROME c

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1. Introduction

The steady-state kinetics of the reaction between ubiquinone cytochrome *c* oxidoreductase and cytochrome *c* have been studied [1] with the isolated reductase complex (cytochrome bc_1, complex III) and several singly-substituted CDNP-lysine cytochromes *c*. This study demonstrated that the interaction domain on cytochrome *c* is located on the front surface of the molecule containing the exposed edge of the heme prosthetic group in the vicinity of lysines 13, 72, 86, 87 and 27 [1,2]. This is essentially the same as the binding domain observed in [3] from the differential chemical reactivities of the lysines of cytochrome *c* in the presence and absence of cytochrome bc_1 and in [4] with singly-modified cytochromes *c* and succinate–cytochrome *c* reductase.

Although it has been assumed generally that cytochrome c_1 is the direct reaction partner for cytochrome *c* [5,6], the possibility that the latter interacts with the iron–sulfur cluster of complex III has not been excluded. However, with the availability of highly purified beef cytochrome c_1 [7] it has become possible to study the reaction directly. As shown below, the interaction domain on cytochrome *c* for cytochrome c_1, defined from a study of the pre-steady-state kinetics with CDNP-lysine 7, 13, 25, 27, 60, 72, 73, 86 and 87 horse cytochromes *c*, is essentially identical to that obtained with complex III. This demonstrates that, as expected, cytochrome c_1 is indeed the physiological reaction partner and that the purified cytochrome c_1 is just as competent as its counterpart in the intact complex.

2. Materials and methods

Cytochrome c_1 was isolated from beef heart according to [7]. The protein as isolated is fully reduced, contains 32 nmol heme/mg protein and shows a single protein band with mol. wt 31 000 upon SDS–polyacrylamide gel electrophoresis. Horse cytochrome *c* [8,9] as well as the CDNP-lysine cytochromes *c* ([10,11] and N.O., D. L. Brautigan, E.M., unpublished) were prepared as described therein. The derivatized proteins are designated by the sequence position of the modified lysine. The mM extinction coefficient (reduced minus oxidized) was taken to be 19.2 for cytochrome c_1 at 552.5 nm [12] and 21.1 for cytochrome *c* at 550 nm [13].

Before use the ferricytochromes *c* were passed through columns (0.7 × 18 cm) of Sephadex G-50 superfine (Pharmacia) equilibrated in 300 mM acetate (Tris) (pH 7.5) containing 1% (v/v) Tween 20, in order to separate any polymeric material. The cytochrome c_1 was similarly treated employing a column of Ultrogel AcA-54 (LKB). The reaction between ferrocytochrome c_1 and ferricytochrome *c* was studied with a modified Durrum-Gibson stopped-flow apparatus with a 2 cm optical pathlength reaction chamber. Since at low ionic strength the reaction is too fast [7], it was studied in the 300 mM acetate (Tris) buffer at 10°C and was followed at 547.5 nm [6] (fig.1).

Abbreviations: CDNP, 4-carboxy-2,6-dinitrophenyl; SDS, sodium dodecyl sulphate

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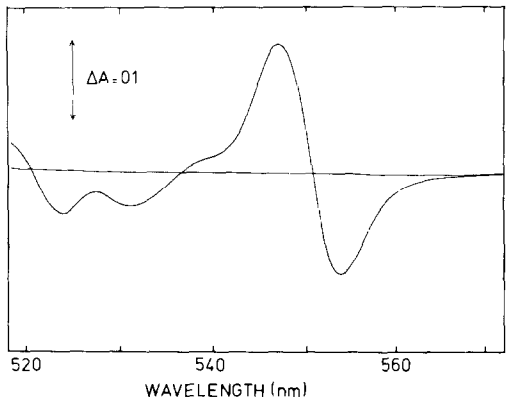

Fig.1. Difference spectrum for the reduction of ferricytochrome c by ferrocytochrome c_1 [$(c_1^{2+} + c^{3+})$ minus $(c_1^{3+} + c^{2+})$]. Before measuring, both the sample and the reference tender cells contained 23 μM ferrocytochrome c_1 and 23.5 μM ferricytochrome c in their respective compartments. After scanning the baseline, the contents of the two compartments of the sample cell were mixed. The difference spectrum was recorded at 20°C in 300 mM acetate (Tris) (pH 7.5), 1% (v/v) Tween 20 with a Cary 17 spectrophotometer.

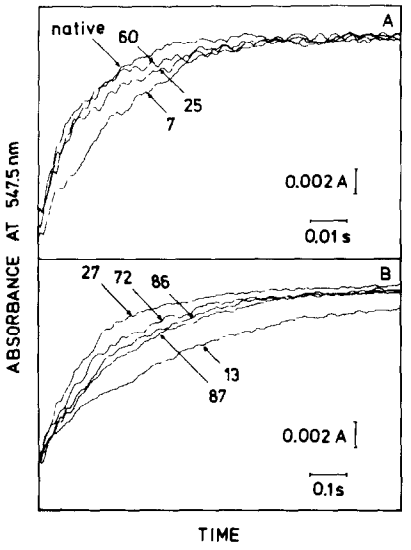

Fig.2. Single traces of the time course of the reaction of ferrocytochrome c_1 (8.0 μM) with native and various CDNP-ferricytochromes c (0.8 μM). See section 2 for experimental conditions. The numbers refer to the sequence position of the modified lysyl residues. The reactions with the more inhibited derivatives (B) are plotted on a time scale which is 10-times larger than used for native cytochrome c and the less inhibited derivatives (A).

The photomultiplier output signal was transferred via a log-converter to a Datalab 905 Transient recorder as a 1024 point data file and stored in a Hewlett Packard 2100A computer. All traces were subjected to a 5-point smoothing procedure [14]. The rate constants were calculated from the initial part of the reaction using a non-linear least-squares best fit [15]. The experimental curves and those corresponding to the rate constants were displayed simultaneously to allow visual inspection of the fit. It was found that a 3-fold excess of cytochrome c_1 sufficed to yield pseudo-first-order kinetics [15], so that the time courses of the reactions studied accurately fit single exponentials. The observed rate constants (k') are mean values of 4–8 traces (SD of 4–6%). The values of k' are plotted against the cytochrome c_1 concentration, the component in excess. The slopes were estimated by a weighted least-squares method and correspond to the second-order rate constants, k_1, with a SD of 6–10%.

3. Results and discussion

Fig.2 shows the time course for the reaction of ferrocytochrome c_1 with the native and CDNP-lysine ferricytochromes c studied. Reliable results were obtained by averaging at least 4 traces. The data plotted in fig.3 demonstrate that the modified cytochromes c differ widely in their reactivities towards cytochrome c_1. The second-order rate constants (table 1) vary over a 40-fold range and increase in the following order: CDNP-lysine 13 <87 <86 <72 <27 <7 <25 <60 <native cytochrome c.

These results are very similar to those obtained [1] for the steady-state kinetics with purified beef complex III, the order of increasing activities being: CDNP-lysine 87 = 86 <13 <72 <27 <25 <7 <60 <native horse cytochrome c. Clearly, the interaction domains on cytochrome c reacting with purified beef cytochrome c_1 and with beef complex III are the same, providing strong evidence that in both cases cytochrome c reacts with the same electron-exchange partner. The minor differences in the activities of the modified cytochromes c observed with the two systems may reflect slight changes in the environment of cytochrome c_1 when purified from the bc_1 complex.

Employing a partially purified cytochrome c_1 preparation and examining the differential reactivities of

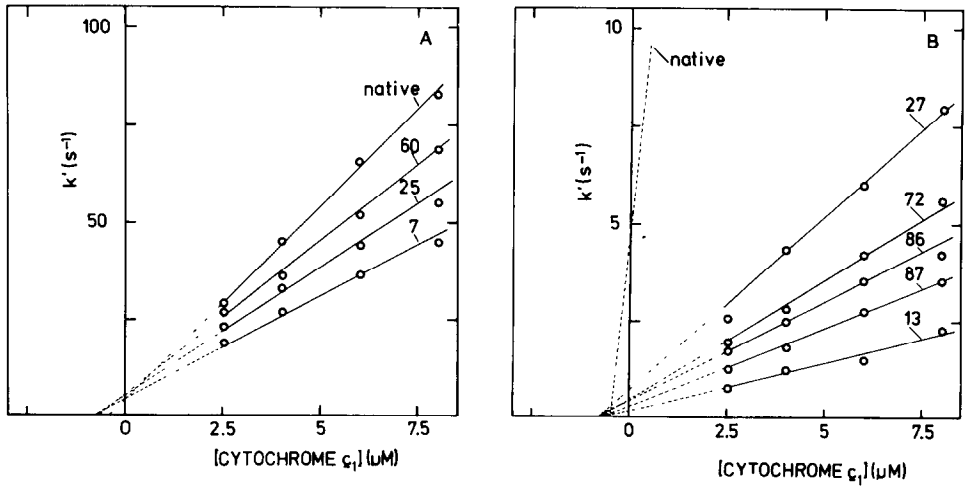

Fig.3. Relationship between the observed pseudo-first-order rate constants (k') and the concentration of ferrocytochrome c_1. The numbers refer to the sequence positions of the modified lysyl residues. In (A) the results with native horse and the more active derivatives are plotted. Native horse cytochrome c is also given for comparison in (B), which shows the results for the more inhibited CDNP cytochromes c.

lysines on horse cytochrome c when free and when complexed with the cytochrome c_1, a binding site similar to the above was deduced [16]. Thus, the interaction and binding domains on cytochrome c for cytochrome c_1 appear to be the same.

Possibly the most significant result of this study is that the properties of the highly purified beef cytochrome c_1 are almost the same as those of cytochrome c_1 in the intact reductase complex, justifying the use of the purified component for extended studies of the mechanism of electron transfer through the respiratory chain.

Table 1
Second-order rate constants for the reactions of ferrocytochrome c_1 with various CDNP-ferricytochromes c[a]

Cytochrome c	$k_1 \times 10^{-5} \pm$ SD ($M^{-1} \cdot s^{-1}$)
Native	100 ± 10
CDNP-lysine 60	80 ± 8
CDNP-lysine 25	60 ± 6
CDNP-lysine 7	50 ± 5
CDNP-lysine 27	9.4 ± 0.6
CDNP-lysine 72	6.5 ± 0.4
CDNP-lysine 86	5.2 ± 0.3
CDNP-lysine 87	4.3 ± 0.3
CDNP-lysine 13	2.6 ± 0.2

[a] The reactions were monitored at 10°C in 300 mM acetate (Tris) (pH 7.5) 1% (v/v) Tween 20

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