KINETIC STUDIES ON CYTOCHROME b–c_1 INTERACTION IN THE ISOLATED SUCCINATE-CYTOCHROME c REDUCTASE

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1. Introduction

The role of cytochrome b_{566} in the energy coupling reactions at site II has been, during the past three years, under constant investigation [1–6]. The most intriguing phenomenon is the anomalous reduction of this cytochrome observed on addition of oxidants to antimycin A-supplemented intact or detergent treated mitochondria and a preparation of succinate-cytochrome c_1 reductase [7–9]. A similar reduction has been observed in the uncoupled mitochondria, in the absence of antimycin A, when the temperature was lowered to about 0° [10]. Wilson et al. [11, 12] observed that in the preparation of succinate-cytochrome c_1 reductase made aerobic in the presence of succinate/fumarate (1:10) the half-reduction potential of cytochrome b_{566} became at least 175 mV more positive when electron transport through cytochrome c_1 was activated. This has been interpreted as an evidence that cytochrome b_{566} is actively engaged in the energy transduction process at site II and that this site remains functional in the preparation of succinate-cytochrome c_1 reductase.

Kinetic studies on the components of site II of the intact mitochondria, especially the b cytochromes, have been carried out recently in great detail [4–6]. This contribution reports kinetic interactions of cytochromes b_{566} and c_1 in the preparation of purified succinate-cytochrome c_1 reductase.

2. Materials and methods

Succinate-cytochrome c_1 reductase was prepared from chicken heart mitochondria essentially by the method of King and Takemori [13] modified as described in [12]. Kinetic studies were carried out using a regenerative flow apparatus [14] mounted on a dual-wavelength spectrophotometer.

The suspension of succinate-cytochrome c_1 reductase in the presence of succinate does not consume oxygen at a significant rate. Thus the removal of oxygen from the incubation mixture was accomplished by the addition of actively respiring *S. cerevisiae* yeast cells supplemented with glucose.

Electron transport was activated by injecting potassium-ferricyanide from the side syringe to the anaerobic suspension of the reductase preparation. In order to avoid oxygen contamination, helium gas was continuously passed through the ferricyanide solution. Under such conditions, the length of the oxidation cycle was proportional to the amount of ferricyanide added.

Protein was determined by the biuret method [15].

3. Results

3.1. *The effect of adding ferricyanide to an anaerobic suspension of succinate-cytochrome c_1 reductase*

The addition of 350 μM K-ferricyanide to an anaerobic suspension of succinate-cytochrome c_1 reductase in the presence of succinate–fumarate (in the ratio of 1:10) induces oxidation of cytochromes c_1

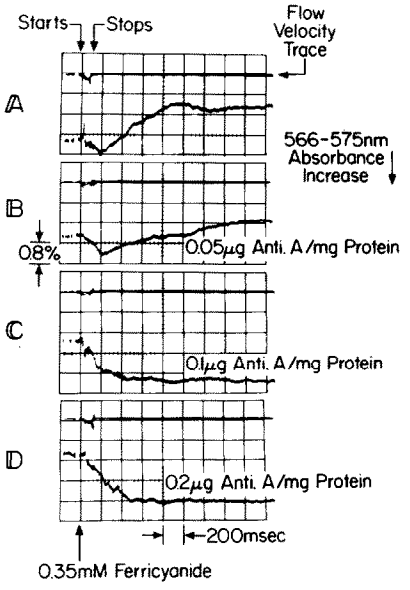

Fig. 1. Behavior of cytochrome b_{566} upon addition of ferricyanide to an antimycin A supplemented preparation of succinate-cytochrome c_1 reductase under anaerobic conditions. Purified succinate-cytochrome c_1 reductase was suspended in 0.01 M phosphate buffer pH 7.0 at a concentration of 1 μM cytochrome c_1. The suspension was made anaerobic by the addition of 1.5 mM succinate and 20 mg wet weight/ml of *S. cerevisiae* yeast cells and 10 mM glucose. After anaerobiosis was attained, 15 mM fumarate was added. Antimycin A concentrations are specified in the figure.

and *b*. Oxidation of *b* cytochrome (fig. 1A) measured at 566 minus 575 nm is preceded by rapid downward deflection of the trace indicative of the transient reduction of this cytochrome. When increasing amounts of antimycin A are added to the incubation mixture, the extent of the reduction phase increases. At a concentration of antimycin A of 0.2 μg/mg protein [trace D], the *b* cytochrome undergoes quantitative reduction when the oxidant is added. Only thereafter a slow reoxidation, invisible on the time-scale of the recording of fig. 1, occurs. Independent spectral studies show that the *b* cytochrome which becomes reduced upon the addition of ferricyanide, is cytochrome b_{566}. Thus in the reductase preparation, cytochrome b_{566} exhibits the same anomalous behavior as it does in the intact mitochondria [7–12].

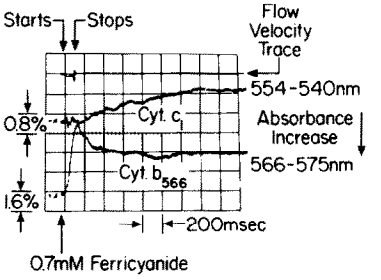

Fig. 2. The correlation between oxidation of cytochrome c_1 and the reduction of b_{566} on addition of ferricyanide to succinate-cytochrome c_1 reductase under anaerobic conditions. Conditions are those of fig. 1. 0.2 μg antimycin A/mg protein was added.

3.2. *Correlations between the oxidation of cytochrome c_1 and the reduction of cytochrome b_{566}*

Reduction of cytochrome b_{566} induced by the addition of oxygen to the anaerobic suspension of pigeon heart mitochondria supplemented with antimycin A [7, 14] is very rapid and occurs with a half-time of about 3–4 msec. i.e., at a rate indistinguishable on a kinetic basis from the rate of oxidation of cytochrome c_1. The time courses of oxidation of cytochrome c_1 and the reduction of b_{566} following the addition of 700 μM ferricyanide to an anaerobic suspension of th reductase preparation (in the presence of antimycin A) is shown in fig. 2. Cytochrome c_1 is mostly oxidized within the 20 msec of the flow time, while the reduction of cytochrome b_{566} occurs with a half-time of about 70 msec and is accompanied by a slower phase of the oxidation of cytochrome c_1 (slow reoxidation of cytochrome b_{566} is not visible on the 200 msec/cm time scale of the recording). Thus in the preparation of the succinate-cytochrome c_1 reductase, in contrast to the intact mitochondria, oxidation of cytochrome c_1 precedes the reduction of cytochrome b_{566} even at 23°.

3.3. *The reduction of cytochrome b_{566} induced by the addition of ferricyanide to an anaerobic succinate-cytochrome c_1 preparation at low temperatures*

It was demonstrated [10] that the reduction of cytochrome b_{566} induced by the addition of oxygen could be observed in the uncoupled mitochondria in the absence of antimycin A when the temperature of

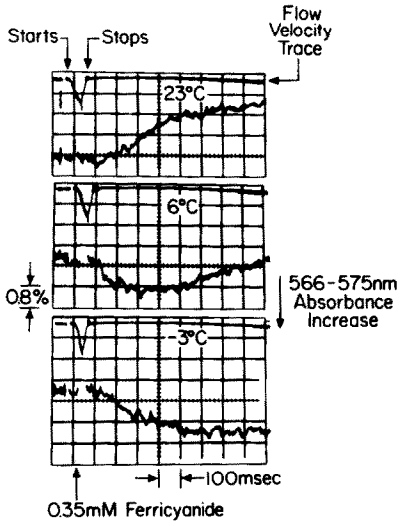

Fig. 3. The effect of temperature on the kinetic behavior of cytochrome b_{566} upon the addition of ferricyanide to the succinate-cytochrome c_1 reductase under anaerobic conditions. Purified succinate-cytochrome c_1 reductase was suspended in 0.04 M phosphate buffer – 30% ethylene glycol pH 6.5 medium in a concentration of 0.8 μM cytochrome c_1. The suspension was made anaerobic by the addition of 2 mM succinate and 40 mg wet weight/ml of *S. cerevisiae* cells and 10 mM glucose. After anaerobiosis 20 mM fumarate was added and the temperature of the flow apparatus lowered as described in [20].

the incubation mixture was lowered to about 0° (or below). A similar reduction of cytochrome b_{566} upon the addition of ferricyanide was obtained in the reductase preparation by Wilson et al. [12] when the temperature of the sample was 5°.

Fig. 3 presents 3 traces recorded at 566–575 nm upon the addition of ferricyanide to the anaerobic suspension of the reductase preparation at various temperatures (23°, 6° and −3°). The curves closely resemble those shown in fig. 1, which were obtained by increasing amounts of antimycin A although under conditions of fig. 3, no antimycin A has been added. As the temperature is lowered, the initially (at 23°) small reduction phase increases in extent. Spectral studies not shown here demonstrate that the cytochrome which is being reduced is cytochrome b_{566}.

At −3° (as at room temp. in the presence of antimycin A) reduction of cytochrome b_{566} is preceded

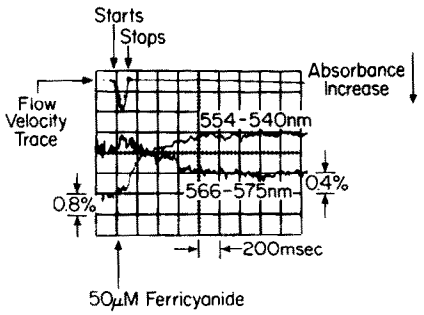

Fig. 4. The relationship between the oxidation of cytochrome c_1 and the reduction of b_{566} on the addition of ferricyanide to the preparation of succinate-cytochrome c_1 reductase at −3°. The conditions were the same as in fig. 3.

by the oxidation of cytochrome c_1. Parallel recordings of the changes following ferricyanide addition at 554–540 nm and 566–575 nm are presented in fig. 4.

4. Discussion

Succinate-cytochrome *c* reductase contains as its most integral part the components involved in the energy conservation at site II, cytochromes *b* and c_1 [1, 3, 4, 17, 18]. An additional advantage in using the reductase preparation lies in the fact that it is freely permeable to both succinate and fumarate, thus by proper adjustment of the ratios of both substrates the redox potential of the reducing couple is easy to calculate.

In the experiments described in this paper the redox potential of the succinate/fumarate was adjusted to +65 mV (ratio 1:10) thus cytochrome b_{566} ($E_{m7.0}$ = 30 mV [1]) remained under anaerobic conditions approx. 97% oxidized. Under such conditions, in agreement with the data of Wilson et al. [11, 12] it was found that cytochrome b_{566} underwent nearly quantitative reduction when the anaerobic succinate-cytochrome *c* reductase was rapidly mixed with the solution of ferricyanide. The effect is not induced specifically by ferricyanide since the same result could be obtaining using cytochrome *c* peroxidase, cytochrome *c* and hydrogen peroxide, or cytochrome oxidase, cytochrome *c* and oxygen as the oxidants of cytochrome c_1 [11].

The most important conclusion which arises from the kinetic studies described in this work is that the rate of aerobic reduction of cytochrome b_{566} in the purified preparation, is rapid enough to be consistent with the phenomenon reflecting the primary energy conservation event at site II. Further, the fact that cytochrome c_1 oxidation precedes the reduction of cytochrome b_{566} indicates that the oxidation of c_1 is the necessary prerequisite for the observed rapid reducibilities of b_{566}.

The aerobic reduction of cytochrome b_{566} is not observed only in the presence of antimycin A. It was shown here and elsewhere [10, 12] that similar results are obtained when the temperature of the sample is lowered. Since the transfer of energy away from the respiratory chain carriers has a higher temperature coefficient [19] than electron transport reactions, lowering the temperature inhibits an energy transfer reaction in a similar way as does the addition of antimycin A. Thus both result in an increase in the steady-state concentration of the primary high energy intermediate at site II.

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