ACCELERATED CATALYSIS BY ACTIVE SUCCINATE DEHYDROGENASE: A REFLECTION OF A NOVEL REGULATORY SITE

Asher GOPHER and Menachem GUTMAN
Department of Biochemistry, The George S. Wise Center of Life Sciences, Tel-Aviv University, Ramat-Aviv, Israel

Received 20 April 1976

1. Introduction

While studying the biphasic reduction of cytochrome *b* in submitochondrial particles [1] we previously considered that the biphasic nature might be associated with the redox state of cytochrome b_{561} or CoQ. In order to investigate this notion we varied the redox potential of the reductant (succinate) by addition of fumarate. It was noted that under certain conditions the presence of fumarate accelerated the rate of reduction of cytochrome *b* in the rapid phase without affecting the second one [2]. As will be shown in this paper the acceleration of cytochrome *b* reduction by fumarate is a reflection of an increase of succinate dehydrogenase activity due to interaction of fumarate (or succinate) with the enzyme in a mechanism distinct from the well known activation [3].

In previous studies, concerning the equilibrium between the active and non active enzyme [4], the active fraction was correlated with measured catalytic activity. As will be shown the activated enzyme is only potentially active and reaches its maximal activity (observed as acceleration) only after interacting with succinate or fumarate. Thus the observed rate of succinate dehydrogenase activity is determined by three sets of parameters: the level of activation, the K_M and substrate concentration and a novel turnover control mechanism. This control reflects binding of succinate or fumarate to a distinct turnover control site. Unless this site is occupied by its ligands, no activity will be measured.

Abbreviations: ETP_H, phosphorylating submitochondrial particles; SDH, succinate dehydrogenase; PMS, phenazine methosulfate; DCPIP, dichlorophenol indophenol; V_∞, the catalytic activity of SDH measured after equilibrated of the enzyme with saturating (5 mM) fumarate; V_i, the catalytic activity of SDH measured during the first 10 sec. following simultaneous addition of succinate, PMS and DCPIP.

2. Materials and methods

ETP_H were made of beef heart mitochondria [5] according to Hansen and Smith [6]. Succinate dehydrogenase was activated by incubating ETP_H in 0.18 M sucrose, 50 mM Tris-acetate, 5 mM $MgSO_4$, pH 7.4 (STM buffer) at 2 mg/ml and 2 mM malonate 30 min 30°C. The ETP_H were spun down and resuspended in STM at 20 mg/ml.

Succinate dehydrogenase activity was measured spectrophotometrically [7] as modified by Gutman et al. [8] using Gilford 240. When most initial rates were studied (V_i), the reaction was carried out at 3°C and the reaction was initiated by the simultaneous addition of succinate (50 mM) PMS (10 mM) and DCPIP (50 μM) using chart speed of 2–5 sec/inch.

Equilibration with fumarate: activated ETP_H in STM buffer were equilibrated at 3°C for 30 min with fumarate. Samples were assayed for initial catalytic activity as described above.

Kinetics of cytochrome *b* reduction and the kinetic analysis was carried out as described before [1] using Aminco-Chance spectrophotometer with response time of 0.1 sec.

 North-Holland Publishing Company – Amsterdam

3. Results and discussion

The reduction of cyt. *b* by succinate at 3°C (antimycin 3 nmol/mg protein and KCN 2 mM present) is depicted in fig.1. Line *A* is a typical reduction pattern consisting of two phases rapid and slow (see insert). Pretreatment of the particles (5 min) by 1 mM fumarate increased the rate of the reduction (line B). If 5 mM fumarate were used, addition of succinate, led to instantaneous reduction (line C). The acceleration by fumarate of the reduction is time dependent. Were succinate and fumarate added simultaneously, the rate of reduction did not reflect the presence of fumarate. Some 30 sec of pre-incubation with fumarate were needed for the effect to reach completion.

The dependence of the rate constants on fumarate concentration is shown in fig.2. To ensure a maximal

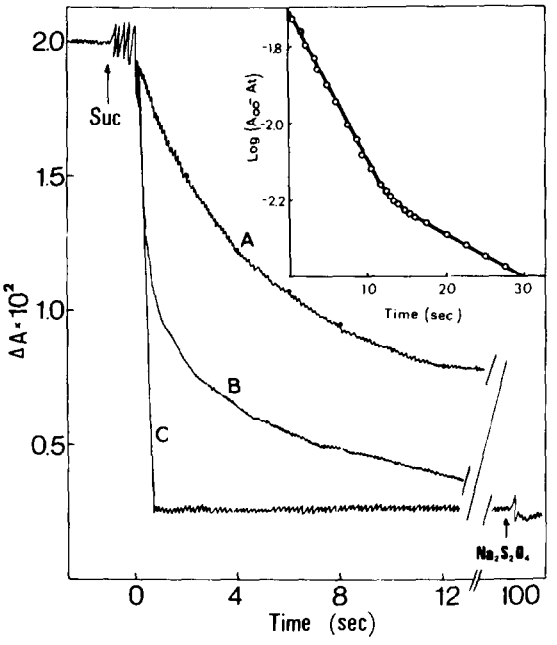

Fig.1. The effect of fumarate on the rate of cytochrome *b* reduction. (A) ETP_H (2 mg/ml at 3°C) were inhibited by 2 mM KCN and Antimycin (3 nmol/mg protein). The reduction started by addition of 60 mM succinate. (B) ETP_H were preincubated with 1 mM fumarate for 5 min before the addition of succinate. (C) ETP_H were treated as in (B) but with 5 mM fumarate. Insert: kinetic analysis of line A showing the rapid and the slow phase [1]. $k_1 = 0.09\ sec^{-1}$ $k_2 = 0.024\ sec^{-1}$.

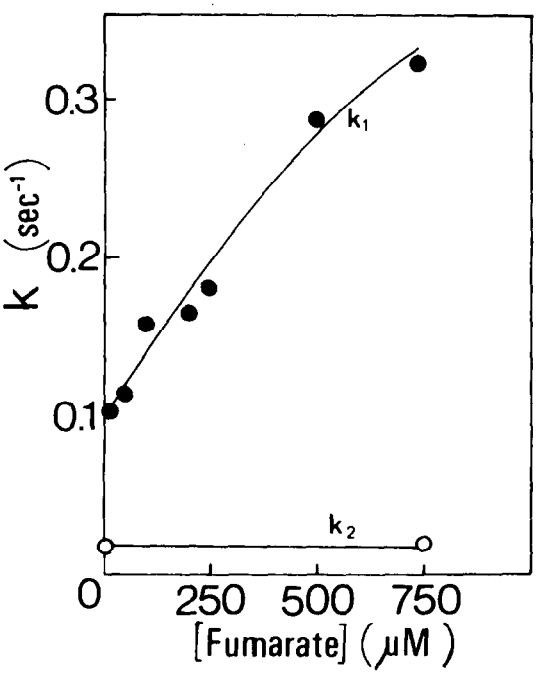

Fig.2. The dependence of rate constants of cytochrome *b* reduction on fumarate concentration. The reaction was measured as in fig.1 using the indicated fumarate concentration. The rate constants were calculated as in insert for fig.1.

effect fumarate was added 5 min before succinate. As seen, the rate of the rapid phase increases with fumarate but the rate of the second phase is constant. It was shown before [1] that the rapid phase of the reduction is limited by the turnover of the dehydrogenase. Thus acceleration of the first phase means an increase in the turnover of succinate dehydrogenase. It was of interest to look whether such acceleration can be observed in uninhibited electron transport too. An increase in turnover of the enzyme should increase succinoxidase activity, but the response time of the oxygen electrode was insufficient for such measurements. Because of that we looked at the effect of fumarate on electron flux by following the steady state level of reduction of *b*, *c* and *a* cytochromes during succinate oxidation. This is a sensitive method for observing variation in partial rate constants. As seen in fig.3, the final steady state of reduction is independent of fumarate, but in its presence the final steady state was reached at a much shorter time.

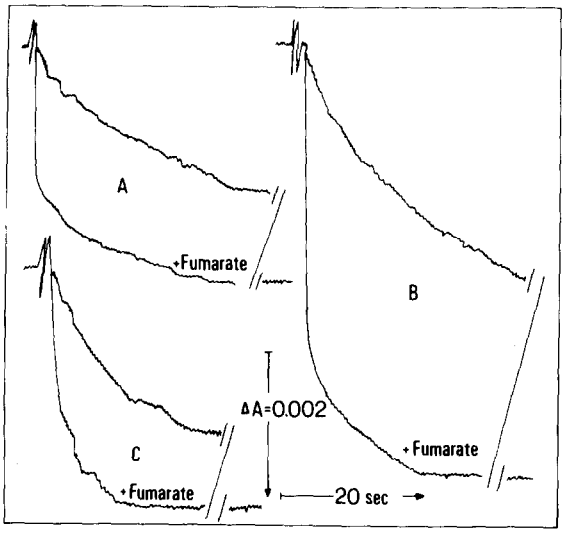

Fig.3. The effect of fumarate on the approach to steady state reduction of cytochromes *b*, *c* and *a*. Experimental conditions as in fig.1, but KCN and Antimycin were omitted. (A) Cytochrome *a* (measured at 605–630 nm). (B) Cytochrome *b* (563 -575 nm). (C) Cytochrome *c* (550–540 nm).

The constancy of the final level of reduction indicates that none of the partial rate constants participating in succinoxidase activity are affected by fumarate. But the slow approach to steady state measured in absence of fumarate indicated that during the pre-steady state phase, the rate of electron flux to the cytochromes increases with time. Thus the enhancement by fumarate of electron flux to the cytochrome system was observed in blocked as well as in open respiratory chain. The most probable component where such enhancement can take place is succinate dehydrogenase. Because of that we looked for acceleration of the catalytic activity of the enzyme. Fig.4, depicts the reduction of DCPIP by succinate dehydrogenase measured at 3°C. Activated and washed ETP_H were kept for 30 min at 3°C in the cuvette (see below), and the reaction was started by simultaneous addition of substrate and electron acceptors (line A). It is evident that V_i is essentially zero and accelerates with time till reaching after ~ 300 sec a maximal steady rate (V_∞). Line B represents the same preparation only the enzyme was equilibrated in the cuvette with 0.25 mM fumarate (30 min 3°C) and the reaction started as above. In

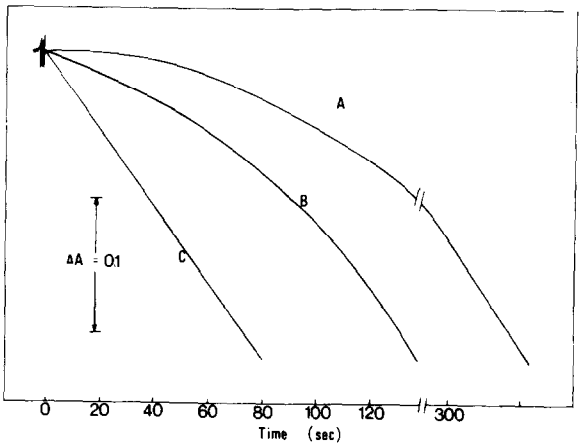

Fig.4. Recorded tracing of succinate dehydrogenase activity measured after preincubation for 30 min at 3°C with fumarate. (A) No fumarate added. (B) 0.250 mM fumarate. (C) 2 mM fumarate. The reaction measured at 3°C was started by simultaneous addition of 60 mM succinate, 10 mM PMS and 50 μM DCPIP as described before.

this case V_i is not zero and the same V_∞ is obtained after a short time. Line C was measured for enzyme treated as above with 5 mM fumarate. In this case the rate is linear and $V_i = V_\infty$. Identical results were obtained with soluble enzyme [9]. The acceleration examplified in line A is a first order reaction (fig.5). The acceleration is relatively slow at 3°C but at 25°C it is 90% over within the first 20 sec.

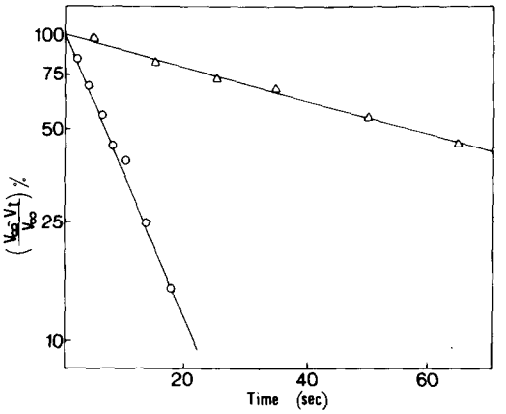

Fig.5. Kinetic analysis of acceleration of succinate dehydrogenase activity by succinate at two temperatures. The reaction was measured as in Fig.4 line A (○) 25°C, (△) 3°C. V_∞ (3°C) was 0.07 μmol/mg min and V_∞ (25°C) was 0.3 μmol/mg min.

The acceleration of succinate dehydrogenase can be caused by either succinate present or fumarate formed during the assay. Considering the amount of fumarate formed (in fig.4, line A when V_∞ is established the concentration of fumarate formed is ~ 0.1 μM), it is most likely that in this case succinate and not fumarate is the effector. On this ground we conclude that both succinate and fumarate can interact with active succinate dehydrogenase in a mode allowing the enzyme to express its catalytic activity. The usage of fumarate allows us to study this transformation separated in time and space from the redox reaction used to monitor the catalytic activity.

We might ascribe the acceleration phenomena to 3 possible mechanisms: (1) Activation of succinate dehydrogenase (2) Reversal of competitive inhibition caused by the malonate used for activation (3) A novel mechanism affecting the catalytic activity of the enzyme.

It is practically impossible to explain the acceleration by the classical activation of the enzyme [2,3]; (a) Enzyme preactivated to 85–95% still exhibit $V_i = 0$ (fig.4, line A); (b) The temperature of the assay (3°C) prohibits any activation in situ; (c) The fumarate concentration at equilibrium $V_i = 0.5\ V_\infty$ (0.35 mM) [9] is significantly lower than that giving 50% activation (5.6 mM) [10] and; (d) The rate constant of acceleration $k_{acceleration} = 0.11\ sec^{-1}$ (25°C) is some 40 times faster than activation $k_{activation} = 0.17\ min^{-1}$ (25°C).

Reversal of inhibition by malonate can also be excluded. The malonate used for activation was removed by the washing procedure and whatever was left in the pellet was further diluted in the enzymic assay. The succinate concentration used in the assay (60 mM) was high enough to displace malonate from the substrate site. Finally, increase of V_i by equilibration with fumarate was observed in preparation activated by NaBr [9] where no malonate was present.

The third possibility assumes that even activated enzyme cannot function unless its turnover control site is occupied by succinate or fumarate. There is a clear difference between K_i (fumarate) 1.1 mM (3°C) and fumarate concentration where $V_i = 0.5\ V_\infty$ (0.35 mM [9]) suggesting that different binding sites are involved. Furthermore, the rate of acceleration is significantly faster than activation but certainly slower than the turnover. It must then represent interaction with a site involved neither in activation nor catalysis – the turnover control site.

Finally it is of interest to discuss why this phenomena was not observed before. The answer to this question is derived from the conditions needed for observing the acceleration: (1) the enzyme should be active but free of succinate or fumarate; (2) the catalytic assay should be measured at a temperature low enough that the acceleration period will be conveniently long. The recommended method for the assay of the enzyme [7] complies with neither of them. The enzyme is activated by succinate and the temperature of the assay is high enough so that acceleration would be too rapid to observe.

References

[1] Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 52, 107–116.
[2] Eisenbach, M. and Gutman, M. unpublished results.
[3] Singer, T. P., Kearney, E. B. and Ackrell, A. B. (1973) Adv. Enzymol. 37, 189–272 Willey, New York.
[4] Gutman, M. (1976) Biochemistry., in the press.
[5] Ringler, R. L., Minakami, S. and Singer, T. P. (1963) J. Biol. Chem. 238, 801–810.
[6] Hansen, M. and Smith, L. A. (1964) Biochim. Biophys. Acta. 81, 214–224.
[7] Singer, T. P. (1974) Methods of Biochemical Analysis 22, 123–175.
[8] Gutman, M., Kearney, E. B. and Singer, T. P. (1971) Biochemistry 10, 2726–2732.
[9] Gopher, A. and Gutman, M. to be published.
[10] Kearney, E. B. (1957) J. Biol. Chem. 229, 363–375.