Are some interactions between NADH oxidase and succinate oxidase in beef heart non-phosphorylating submitochondrial particles artifacts?

M. Miranda, D. Botti and C. Pantani[1]

Institute of General Biology, L'Aquila University, via Assergi 6, I–67100 L'Aquila (Italy), 4 October 1976

Summary. Critical review of literature and experiments by the authors are suggestive that some interactions between mitochondrial respiratory complexes could be due to a minimal matrix enzymes contamination.

Aerobic succinate oxidation has been shown to stimulate acetoacetate reduction[2,3]; this process is not an energy-linked, succinate-dependent NAD^+ reduction[4,5]. On the other hand, rat liver submitochondrial phosphorylating particles can carry out an energy linked NAD^+ reduction sustained by succinate oxidation[6,7]. Moreover, interactions between complex I and II, mediated by complex III, have been demonstrated to occur in beef heart non-phosphorylating submitochondrial particles[8].

Submitochondrial preparations from various materials are widely used to study respiratory complexes and their interactions, but the contamination by mitochondrial matrix enzymes, tightly bound to inner membrane, has often been undervalued, though it has been already pointed out[9,10] that one should be careful when using Keilin-Hartree beef heart muscle preparations, because, due to fumarase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37) contaminations, oxaloacetate will produce from succinate in the presence of NAD^+ and inhibit succinate dehydrogenase (EC 1.3.99.1). In addition, many workers[11–13] have clearly demonstrated that submitochondrial particles from beef heart, rat liver or pig heart, at advanced stage of purification, retain malate dehydrogenase and other matrix enzymatic activities as well. Moreover, it must be pointed out that mitochondrial malate dehydrogenase activity is sometimes assayed under inhibitory oxaloacetate concentrations[12], if compared to those of Englard and Siegel[14], and may, therefore, be undervalued.

Davis et al.[15], who found an inhibitory interaction between succinate oxidase and NADH oxidase in beef heart non-phosphorylating submitochondrial particles, do not mention, or take into account, matrix enzymatic activities of their preparation which, according to Hatefi and Lester[16], oxidizes malate, pyruvate, 2-oxo-glutarate, isocitrate, glutamate and succinate. Purified succinate dehydrogenase has been shown to be inhibited by oxaloacetate with a K_I of $1.5–4.5 \times 10^{-6}$ M[17–20]. This implicates that within integrated mitochondria succinate dehydrogenase should always be inhibited, according to the levels of oxaloacetate found[21]. But it was suggested that oxaloacetate does not interfere with succinate oxidation due to compartmentation[22], oxaloacetate decarboxylase (EC 4.1.1.3) activity and oxaloacetate removal[23–26].

Specific oxidasic and enzymatic activities of beef heart non-phosphorylating submitochondrial particles

	Oxygen monitor natoms O_2 consumed (mg protein/min)	Spectrophotometer nmoles NAD oxidized or reduced (mg protein/min)
Succinate oxidase	47.1	
Succinate oxidase plus cytochrome c	199.8	
NADH oxidase plus cytochrome c	50.3	
NADPH oxidized plus cytochrome c	7.4	
Fumarate plus NAD^+	2.1	
Malate dehydrogenase		56.0
Succinate plus NAD^+ and rotenone		14.2

For reaction mixtures see the text.

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The present work demonstrates that the interaction between NADH oxidase and succinate oxidase in beef heart non-phosphorylating submitochondrial particles, prepared according to King[27] and furtherly purified, can be ascribed to oxaloacetate, though King claims that his preparation 'does not catalize Krebs cycle reactions'.

Material and methods. Beef heart non-phosphorylating submitochondrial particles were prepared following King's method 1[27] and furtherly purified by resuspending pellets in 20 mM potassium phosphate containing 0.1% Na-deoxycholate at pH 7.40. Suspensions were centrifuged at 105,000 × g for 60 min in a SW50 rotor with a L50 Spinco Beckman centrifuge. Pellets were resuspended in the same medium and centrifuged at 105,000 × g for 75 min in the same rotor. Submitochondrial particles were suspended in 0.10 M potassium phosphate containing 0.1% Na-deoxycholate at pH 7.40. All operations were carried out at 0–4 °C. Proteins were detected by biuret method on samples treated with 1% Na-deoxycholate.

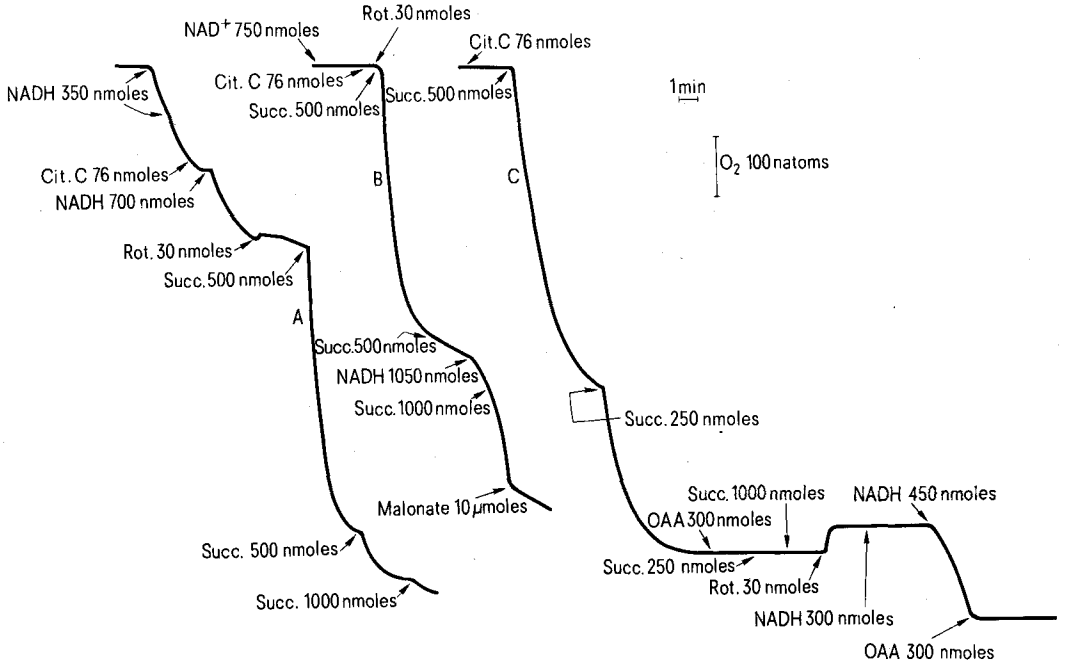

Fig. 1. Oxygraphic curves showing *A* NADH oxidation, rotenone sensitivity and delayed succinate oxidase inhibition; *B* succinate oxidase inhibition by NAD+ and removal of inhibition by NADH; *C* succinate oxidase inhibition by oxaloacetate and removal of inhibition by NADH.

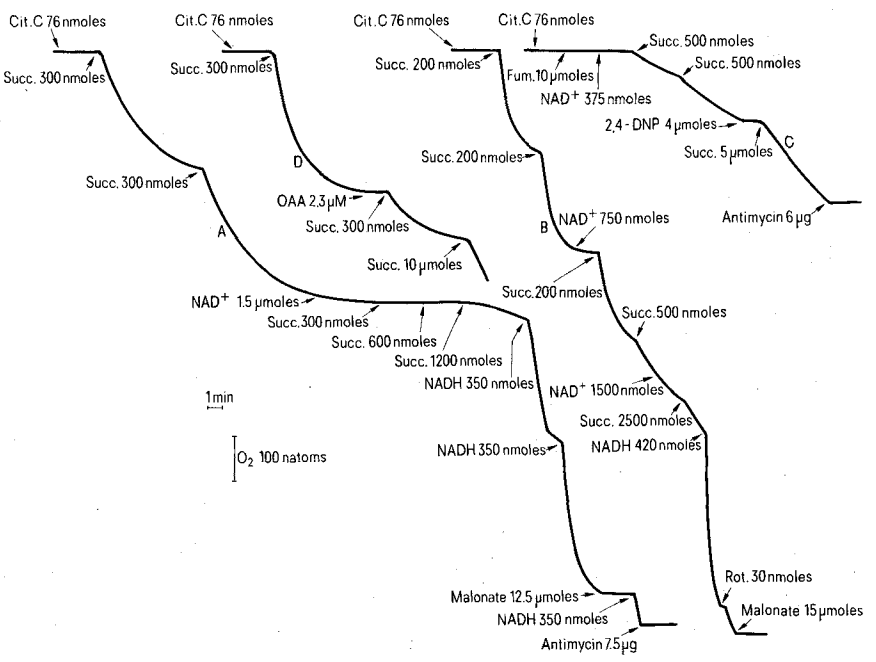

Fig. 2. Oxygraphic curves showing *A* rapid succinate oxidase inhibition by NAD+; *B* succinate oxidase sensitivity to antimycin; *C* action of fumarate plus NAD+ on succinate oxidase activity; *D* inhibition of succinate oxidase by oxaloacetate in the range of K_i for purified succinic dehydrogenase.

O_2-consumption was detected by an YSI oxygen monitor at 25 °C in a reaction mixture containing in a final volume of 3.10 ml: 10 μmoles of $MgCl_2$, 2.25 mg of proteins and 310 μmoles of potassium phosphate at pH 7.40. Reaction mixtures for malate dehydrogenase and succinate dependent NAD^+ reduction were made according to Englard and Siegel[14]. Reagents were purchased from Sigma and Boehringer.

Results and discussion. The characteristics of our preparation are shown in the table. It is evident that malate dehydrogenase specific activity is very low if compared to more sophisticated preparations[11–13].

Figure 1A shows that a) our preparation oxidizes NADH and this oxidation is rotenone-sensitive; b) 500 nmoles of added succinate are stoichiometrically oxidized, but further added amounts are not. The inhibition, as figure 1B demonstrates, is due to NAD^+ generated during NADH oxidation. In fact, succinate oxidase is inhibited by NAD^+ when rotenone is added before succinate. The addition of NADH removes this inhibition, but, due to rotenone presence and malonate sensitivity (figures 1B and 2A), consequent O_2-uptake can only be attributed to succinate and not to NADH. It is likely that, in the presence of NAD^+, oxaloacetate forms from succinate, due to fumarase and malate dehydrogenase contamination (table), and this results in succinate oxidase inhibition. The addition of NADH, in the presence of rotenone, shifts oxaloacetate toward malate, as shown in figure 1C, so removing the inhibition which is repristinated by further oxaloacetate.

Successively added succinate amounts are stoichiometrically oxidized, as shown in figure 2A, but soon after NAD^+ addition O_2-consumption decreases and succinate oxidase activity becomes inhibited. If NAD^+ is added to the reaction mixture before succinate (figure 1B), it will take some time before succinate oxidase be inhibited, possibly because fumarate and malate levels must increase before oxaloacetate is produced. The preparation is antimycin-sensitive with respect to NADH and succinate oxidations (figures 2A and 2C), which is suggestive of complex I, II and III interaction. The finding that NAD^+ inhibits succinate oxidase when rotenone is present (figure 1B) and that NADH removes inhibition in the presence of rotenone (figures 1B and 1C) would rule out Davis's et al.[15] claim that 'both NADH and succinate inhibit the rate of oxidation of the other' by competing for a common respiratory assembly. This supports the suggestion that some of the interactions observed in beef heart non-phosphorylating submitochondrial particles could be artifacts due to matrix enzymes contamination. Moreover, Davis et al.[15] found that NAD^+ does not lower succinate oxidase activity, perhaps because the authors take into account only initial oxidative rates and not what occurs in the time; in fact we have shown that NAD^+ inhibits succinate oxidase only after some lapse of time (figure 2B).

Figure 2C demonstrates that if fumarate and NAD^+ are added to the reaction mixture before succinate, succinate oxidase is early inhibited. Then the quite undetectable O_2-consumption should be enough to produce oxaloacetate to such an extent as to be inhibitory for succinate oxidase. The inhibition by NAD^+ does not appear to be energy-linked, since 2,4-dinitrophenol does not remove it (figure 2C); only the addition of a large succinate amount removes inhibition, as if it were competitive, and the oxidation becomes antimycin-sensitive. Figure 2D shows that the addition of oxaloacetate at a concentration of 2.3×10^{-6} M, that is in the range of K_i for purified succinate dehydrogenase[17–20], strongly inhibits our succinate oxidase preparation, and this supports the view that the preparation is purified enough and not compartmented. On the basis of the findings here reported, and of the literature cited, we would suggest that some interactions between complex I, II and III in submitochondrial particles could also be explained by low fumarase and malate dehydrogenase contaminations. Otherwise, if the findings here reported cannot be explained on the basis of the very low oxaloacetate levels that can form in the reaction mixture, we must conclude that some inhibitory interaction may occur between complex II and NAD^+, and that this inhibition is overcome by NADH in the presence or absence of rotenone and is not energy-linked.

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Paratopic interaction, a mechanism in the generation of structure bound enzymatic activity

H. C. Hemker and H. L. L. Frank[1]

Department of Biochemistry, Biomedical Centre, State University Limburg, NL-Maastricht (The Netherlands), 27 December 1976

Summary. A general mechanism is recognized that can cause specific enzymatic activity at interphases. It consists of 2 proteins bound in close juxtaposition at a micelle or membrane surface. One, the enzyme *sensu strictu*, bears the active site, the other, the paraenzyme, is essential for generation or specific modification of the enzymatic activity.

It is the purpose of this report to draw attention to a kind of interaction between protein molecules and an interface, that can regulate, or even generate, enzymatic activity. The basic unit of this concept consists of 2 different protein molecules adsorbed next to each other onto an interface. This configuration constitutes an enzymatically active moiety. The active site is present on one of the 2 molecules, called the active site carrier; the enzymatic activity, however, is governed by the presence of the second protein molecule, called the paraenzyme. For this kind of interaction we suggest the name

1 Present address: Department of Cardiology, St. Annadal Hospital, Maastricht, The Netherlands.