THE KINETICS OF FLAVOPROTEIN AND PYRIDINE NUCLEOTIDE OXIDATION IN CARDIAC MITOCHONDRIA IN THE PRESENCE OF CALCIUM

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Received 19 June 1972

1. Introduction

The developing interest in the function of mitochondria in calcium metabolism of cells and tissues [1–3] has led us to develop a method for evaluating the calcium environment of mitochondria *in situ* [4]. Hitherto the response of the various respiratory carriers to ADP + P_i and to calcium has been found to be similar [1,5], for example, in terms of the half-time for the oxidation of cytochrome *b* in the resting–active transition (state 4 to state 3) [1]. However, Ca^{2+} caused a greater response of the oxidation-reduction level of NADH than of cytochrome *b* [5]. This paper follows this evidence of a Ca^{2+} specific response from the kinetic point of view; the speed of transition from anaerobiosis to aerobiosis of reduced pyridine nucleotide (PN) and flavoprotein (Fp) is studied and found to be significantly more rapid in the presence of Ca^{2+} than ADP. These results are applied to the study of cytosolic calcium levels in cells and tissues elsewhere [4,6].

2. Materials and methods

Pigeon heart mitochondria [7] were suspended in 0.2 M mannitol sucrose medium buffered with 50 mM morpholinonaphthalene sulphonate (MOPS) pH 6.7 at 1–3 mg/ml. The chemicals used were reagent grade; ADP was obtained from Sigma Chemical Company. Recordings of Fp and NADH kinetics were made in the regenerative flow apparatus with and without liquid dye laser (LDL) activation [8,9]. The preparation is introduced into the flow apparatus, supplemented with substrate and soon becomes anaerobic. Oxygen pulses at a final concentration of 17 μM reactivate electron flow and allow an observation of Fp–PN kinetics under the various conditions. The quality of the preparations was monitored in the flow apparatus by the effect of ADP + P_i upon the oxidation rates of Fp and PN. Fig. 1 shows ratios of 7–10 in the rate in state 3 to that in state 4 [1,5]. The ADP and Ca^{2+} are added directly to the main syringe of the flow apparatus. Since oxygen is reduced to water after each oxygen pulse, repetitive pulsing of the system with oxygen after each addition of ADP and Ca^{2+} is possible. The kinetic responses are recorded on an oscilloscope. Fluorescence signals of reduced PN are obtained by reflectance from the 22 X 22 mm or the 6 X 22 mm faces of the observation chamber [8]. Fluorescence of Fp is recorded by illumination of the 6 X 22 mm side with excitation light at 460 nm and measurement of the emission from the 22 X 22 mm face of the observation chamber at approx. 580 nm [10–15]. In fig. 3B a portion of the mitochondria in the major syringe of the apparatus is equilibrated with carbon monoxide and mixed with the remainder of the contents of the apparatus. Upon injection of oxygen, the mixture of CO-inhibited mitochondria is photolyzed by the 0.1 joule, 585 nm flash of the liquid dye laser (LDL). Cytochrome a_3 then reacts rapidly with oxygen and causes the rapid oxidation of Fp and PN. Their responses are read out as described above. The reaction rates are measured in terms of half-times or in terms of the initial slope as a fraction of the total amplitude per unit time. Thus a high accuracy of the measurement of relative reaction rates is afforded and the problems of absolute calibrations of the fluorometric techniques are not involved.

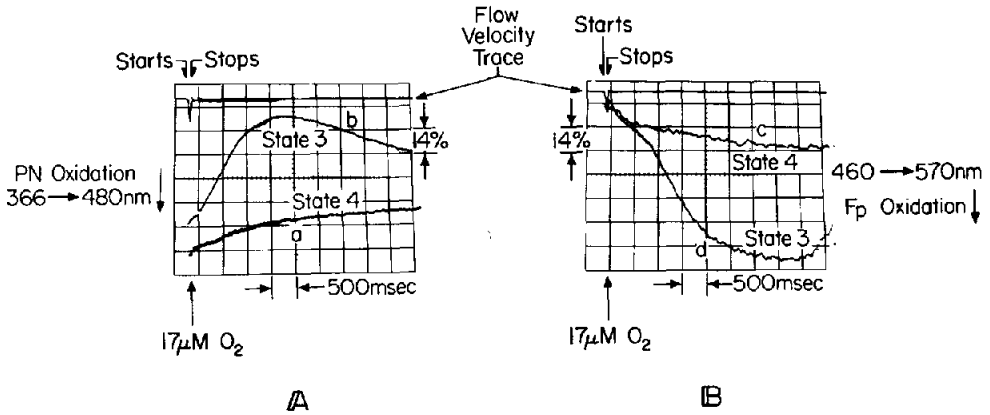

Fig. 1. Illustrating the effects of ADP + P_i upon the responses of PN and Fp by the delivery of oxygen pulses to the anaerobic mitochondria (Expt. 238-1).

When such calibrations are required, they are made by simultaneous measurements of the flavoprotein fluorescence and absorbance under conditions where time or redox potential resolution are possible [11,12]. The temperature of all experiments is 23°–24°.

3. Experimental results

3.1. *Effect of ADP + P_i.*

Fig. 1 illustrates the effects of ADP + P_i upon the response induced in PN and Fp kinetics by oxygen pulses. The control experiment is the transition from anaerobiosis to state 4, no ADP + P_i being added. This is illustrated by the traces a and c of fig. 1, which show

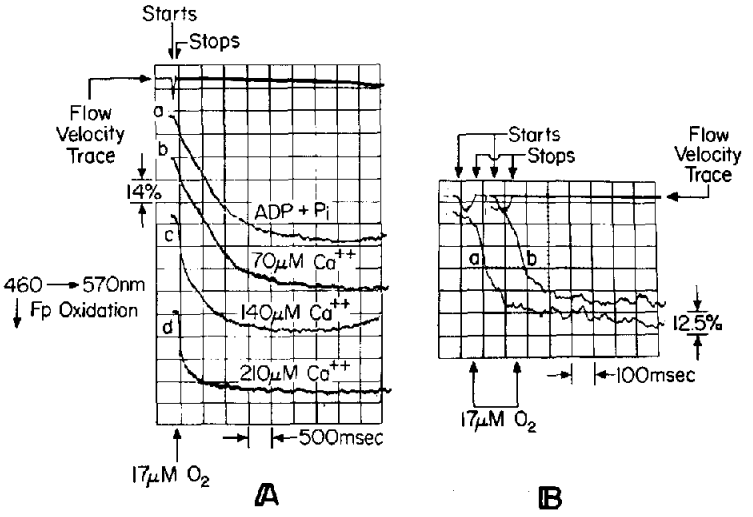

Fig. 2. A) The effect of calcium upon the Fp response. ADP + P_i are present in trace a and increasing amounts of calcium present in traces b, c and d. (Expt. 240). B) Illustrating the rate of oxidation of Fp and the lack of its inhibition by the addition of rotenone. (Expt. 252-2,3).

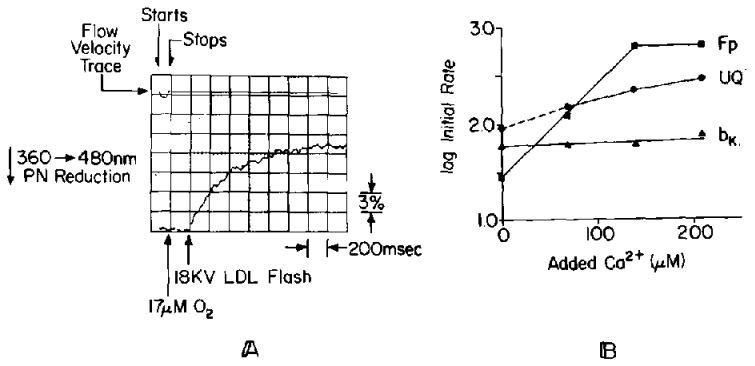

Fig. 3. A) The kinetics of PN oxidation in the presence of calcium. Conditions as in previous figures except that the reaction is initiated by the photolysis of cytochrome oxidase, a_3 –CO compound in the presence of oxygen. (Expt. 359-V).

B) Effect of added calcium upon the oxidation rate of cytochrome b_K ubiquinone and flavoprotein in anaerobic–aerobic transitions. The initial point for the curves (calcium equals zero) corresponds to state 3 (5 mM phosphate, 4.2 mM ADP). In the case of ubiquinone, the ADP level is approx. 10^{-5} due to the high absorption at high ADP concentrations. The protein concentration of pigeon heart mitochondria was approx. 2 mg/ml. The temperature is 24°. The rates are given in nmoles of UQ or b_K per mg protein per minute. In the case of flavoprotein, the scale is in units per sec per mg protein where one unit is equal to a fluorescence intensity change of 25%. (Expt. 240, 241, 242 and 244).

that the oxidation rate is slow except for a small initial jump in Fp. 5 mM ADP and phosphate are then added to the anaerobic mitochondria and the oxygen pulse experiment is repeated as illustrated by traces b and d. The latter transition is from anaerobiosis to state 3 and the ratio of the slopes in state 3 to state 4 at one second after adding oxygen is 7-fold for PN and 10-fold for Fp, the half-times being 0.6 and 1.2 sec, respectively, for the total excursion. The slow kinetics of oxidation of the low potential flavoprotein show clearly in fig. 1B [11,12] since adequate time was permitted to allow reduction of Fp_L in the interval between O_2 injections.

3.2. *Effects of calcium upon the Fp response*

Initial rates of Fp oxidation greatly exceeding those indicated by fig. 1 are obtained by supplements of calcium. In fig. 2A, trace a, a different preparation shows a rapid response in state 3 that does not include the slow kinetics of the low potential flavoprotein of fig. 1. Traces b, c, and d illustrate the effects of supplements of calcium and indicate an over 10-fold acceleration of the Fp slope over the state 3 value at 210 μM calcium. The half-time is evaluated in fig. 2B. The amplitude of the fluorescence increment in the oxygen pulse experiment diminishes as the calcium concentration is increased because of a decrease in the reduction of Fp in anaerobiosis prior to the pulse of oxygen.

3.3. *Rate of Fp response and the effect of rotenone*

Fig. 2B illustrates on expanded time scale the rate of Fp oxidation in the oxygen pulse experiments. The half-time is 60 msec at 210 μM Ca^{2+} (trace a). If rotenone is added, and the experiment is repeated with a second oxygen pulse, the magnitude and the rate of Fp oxidation is the same (trace b). These results are interpreted to indicate that the high potential, rotenone insensitive Fp [10–12] is involved in the fast oxidation reaction and that the low potential, slowly oxidized Fp_L [10] is not.

3.4. *The speed of the PN response*

Fast responses are also observed when the kinetics of oxidation of reduced PN is recorded in calcium supplemented mitochondria (fig. 3A). The half-time for PN oxidation is 200 msec, 3-fold more rapid than those of fig. 1 for the ADP supplemented case. In addition to illustrating the effect of calcium upon the PN kinetics, this chart shows that this rapid response can be measured by flash photolysis of cytochrome a_3 –CO in the presence of oxygen, a technique that is used in the study of the responses of both PN and Fp in intact organs [4,6].

3.5. *Responses of other components*

Experiments of the type of fig. 2 have been repeated for PN, ubiquinone [16] and cytochrome *b* [17]. The logarithms of the rates of the responses are computed as described in the legend and are plotted against the concentrations of calcium in fig. 3B. Only small increases for the rate of cytochrome b_k and ubiquinone are observed. The increase of the Fp rate is ∿10-fold over the ADP + P_i rate and is maximal at about 140 μM Ca^{2+}.

4. Discussion

The evidence presented in this paper identifies a rate difference in the response of Fp and PN to calcium as compared with ADP. Since this observation has direct applicability to the identification of high calcium levels in contact with mitochondria *in situ*, the communication is directed mainly towards the phenomenological aspects. However, certain features of the data are discussion worthy and a preliminary interpretation of the reaction mechanism is provided.

4.1. *The identity of Fp*

Many flavoproteins and iron proteins are identified in the redox potential region between −300 and 0 mV [18–22]. Their absorption bands may overlap, but the fluorescence properties of the flavoproteins distinguish them from the iron proteins. The rapidly responding Fp observed here is localized on the oxygen side of the rotenone site in the respiratory chain by the maintenance of its rapid response to oxygen pulses in the presence of rotenone, and is thereby identified with Fp_{D_2} or Fp_F, the high potential fluorescent Fp [11, 12, 18, 19]. Fp_{D_2} was localized between site I and cytochrome *b* [10] on the basis of studies in intact mitochondria and was later shown to have corresponding fluorescence and absorbance changes in anaerobic–aerobic transitions at wavelengths 470–500 nm ($t_{1/2}$ ≈0.4 sec in the absence of Ca^{2+} [19]). Time resolved excitation difference spectra for pigeon heart mitochondria in the interval 20 msec to 1 sec showed a characteristic excitation band in the region of 500–450 nm appropriate to the flavoprotein Fp_{D_2} [22]. The term Fp_F was used in that communication to designate it as a rapidly responding flavoprotein and to avoid confusion with other nomenclatures of the flavoproteins [22]. In the following, the simple term Fp is used since our attention is focussed on one species.

Initially, the kinetics of fluorescent flavoprotein were recorded in the 50 msec time range [14] employing 436 nm excitation which under these conditions showed a contribution of cytochrome *b* absorbancy changes in the kinetic traces. Employing 470 nm for excitation, no cytochrome *b* interference is observed as indicated by the time resolved excitation spectra [12, 15,19]. The time resolved studies further showed that this fast, rotenone-insensitive flavoprotein contributed 15% to the total fluorescence change in oxidation cycles where the low potential flavoprotein of the lipoate dehydrogenase (Fp_L) was also oxidized.

The observation that the half-time for the oxidation of Fp in the presence of calcium shortens to a value comparable to that of cytochrome *b* in the presence of ADP or calcium suggests that either the rate of reduction of Fp is decreased or its rate of oxidation is increased. Considering that the pathway for the reduction of Fp is through the rotenone-sensitive site, and that when rotenone is added to the calcium supplemented mitochondria that no increase in the rate of Fp oxidation is observed, the apparent acceleration of the rate of Fp oxidation is not due to a decrease in its rate of reduction. Thus, an appropriate kinetic explanation is that its rate of oxidation is increased. Since both thermodynamic and kinetic studies are consistent with cytochrome b_T as the electron transfer component acting across the antimycin A site, and since the acceleration of the rate of cytochrome *b* oxidation by calcium in the presence of ADP does not occur, the interaction site for calcium is presumably between cytochrome b_T and Fp [17].

The likelihood of an energetic interaction of Ca^{2+} and Fp is remote since Fp is not a 'transducing' cytochrome such as cytochrome b_T capable of providing energy necessary for Ca^{2+} accumulation. A more likely explanation of the altered Fp kinetics is that high Ca^{2+} concentrations alter the structural organization of the membrane, and afford an altered environment and an altered pathway for the oxidation of Fp. High concentrations of calcium can alter the charge density distribution of the region of the head groups of the phospholipid components, for example, as is already indicated by the alteration of ANS fluorescence in the presence of divalent cations [23]. These changes could bring Fp_F into more effective contact with its oxidant.

Acknowledgements

The author thanks M. Erecinska for help in the experiments and Keiko Sera for mitochondrial preparations. The work was supported by USPHS GM 12202-08.

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