COMPARISON OF FLUORESCENCE PROBE AND LIGHT-SCATTERING READOUT OF STRUCTURAL STATES OF MITOCHONDRIAL MEMBRANE FRAGMENTS

Britton CHANCE and Chuan-pu LEE
Johnson Research Foundation, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104, USA

Received 30 June 1969

1. Introduction

The possible functional relationship between energy conservation reactions and mitochondrial structure as observed electron microscopically [1] or by non-specific light scattering changes (NSLS) is obscured by kinetic discrepancies between the energy coupling reaction as indicated by cytochrome responses [2], by NSLS [3] and by the "orthodox-condensed" matrix transition of intact mitochondria [4]. Submitochondrial particles afford a simpler system for studying conformational changes as read out by NSLS since they have lost matrix material. In these particles, energy coupling reactions, as read out by the membrane-bound fluorochrome, 8-anilino-1-naphthalene sulfonic acid (ANS) [5], are closely identified with the energy conservation reactions of membrane fragments, as indicated by observations of reversed electron transfer [6] and by titrations with uncoupling agents [7]. Packer has reported energy-dependent NSLS changes in digitonin particles [8] but such preparations have a "sidedness" identical to intact mitochondria [9]. In this communication, well-defined membrane fragments [10] have been employed, in which the subunits of the inner membrane, identified as the coupling factor F_1 [11] project outwards. pH-induced changes of light-scattering, optical rotatory dispersion, and ANS fluorescence have recently been correlated by Packer [12]. In the studies reported here, the response of NSLS as an indicator of membrane conformation is compared with the response of ANS fluorescence as an indicator of energy coupling in transitions between the non-energized and energized states of the membrane. We find that the two phenomena can be both quantitatively and qualitatively distinguished on the basis of the selective effects of permeant anions, energy coupling with oligomycin, and uncoupling with pentachlorophenol.

2. Materials and methods

EDTA particles derived from "heavy" beef heart mitochondria were prepared as described previously [13].

The optical arrangement for measuring ANS fluorescence, NSLS, and cytochrome *a* abosrbancy changes is afforded by fluorescence excitation at 366 nm and fluorescence emission measurement at 450 nm, coupled with dual wavelength measurement of cytochrome *a* at 605–620 nm, the cytochrome being recorded at the shorter wavelength, while the longer wavelength is very close to an isosbestic point in the oxidized-minus-reduced spectrum [2]. Forward scattering of the light at 620 nm is measured by a 17 mm diameter photocathode placed 30 mm from the rear surface of a 1 cm optical path cuvette, conditions similar to those used by us [3] and Tedeschi [14]. Wavelength scanning from 640 to 460 nm showed negligible specific absorbance change caused by the addition of nitrate to succinate and oligomycin supplemented ESMP, verifying that NSLS changes are measured at 620 nm. The dual wavelength-single wavelength signals were distinguished one from anohter by electronic choppers which performed the usual operations of the dual wavelength system and in addition selected out a portion of the alternating waveform corresponding to the 620 nm signal, com-

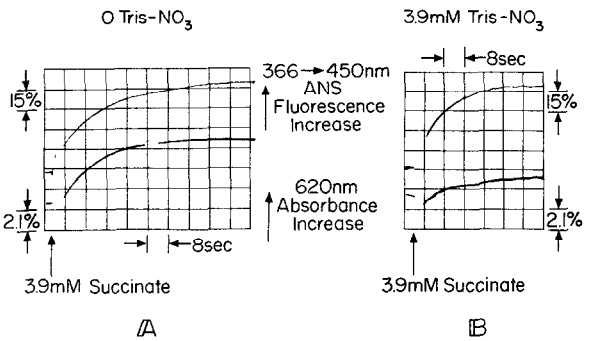

Fig. 1. Effect of permeant anions upon ANS and NSLS changes in membrane fragments. Submitochondrial particles derived from beef heart (ESMP), 1.5 mg of protein per ml, in 0.3 mannitol sucrose, 20 mM Tris-Cl, pH 7.4, supplemented with oligomycin (2 μg/mg particle protein) and 15.3 μM ANS. A, without Tris-nitrate; B, in the presence of 3.9 mM Tris-nitrate.

pared it with a fixed reference voltage, and afforded an amplification of the difference. These outputs are continuously monitored on strip chart recorders having curvilinear coordinates, and experiments requiring a rapid time scale were recorded on a storage oscilloscope.

3. Results

Fig. 1 illustrates, in A, a control experiment in which an increase of fluorescence is caused by succinate activation of electron transport in oligomycin and ANS-supplemented ESMP (OESMP). Time proceeds from left to right in both diagrams. The initial portion of the trace represents the de-energized state of the membrane. Addition of 3.9 mM succinate activates electron transport and energy coupling leading to increased ANS fluorescence. There is a very nearly simultaneous increase of NSLS. The ANS fluorescence trace, however, has a slow "tail" and thus has a longer half-time, 13 sec as compared with 9 sec. Since the ANS reaction involves a transition in the environment of ANS molecules already bound [15] and the binding of additional ANS as well [16], the initial portion of the ANS kinetics more accurately portrays the kinetics of membrane energization than does the latter portion.

If the experiment is repeated with a supplement of 3.9 mM Tris-nitrate, pH 7.4, the NSLS response is reduced more than 50% while the ANS response is reduced only 7%. In the presence of nitrate, the ANS response is more rapid than in the absence of nitrate; the half-time is 8 sec. Furthermore, there is no evidence for a slow phase of the ANS response. The residual portion of the NSLS response follows that of the ANS response up to the half-time and then clearly lags behind it. At higher Tris-nitrate concentrations, the NSLS change is completely eliminated. Since similar results are obtained with iodide, thiocyanate and perchlorate, this experiment shows that the ANS and NSLS changes are quantitatively resolvable by the use of permeant anions.

3.1. *Oligomycin response in the presence and absence of permeant anions*

Fig. 2 illustrates, in A, the response of ANS and NSLS as well as the steady state of cytochrome *a* in ESMP prior to a supplement of oligomycin. On activation of electron transport by adding succinate there is a small fluorescence increase due to the partial coupling of the particles, and a slower and more persistent increase in NSLS. Upon addition of oligomycin, both traces rise very nearly in synchrony, to give maximal values of the energy-dependent ANS fluorescence increase and the NSLS change.

In fig. 2B, 3.9 mM Tris-nitrate is added prior to the activation of electron transport by succinate. On

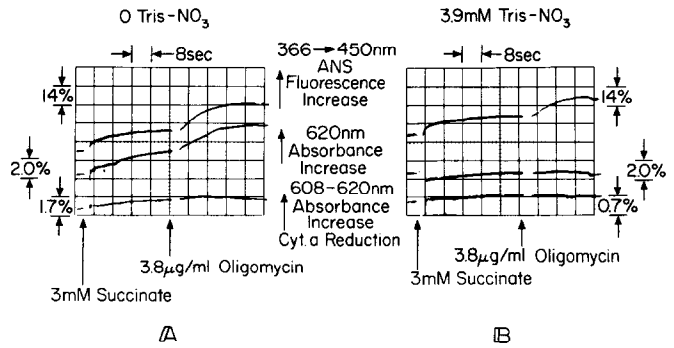

Fig. 2. Effect of permeant anions on the response to oligomycin. ESMP under the conditions of fig. 1 except that oligomycin was not added prior to the addition of succinate; A, without nitrate; B, in the presence of 3.9 mM Tris-nitrate. Top trace, ANS fluorescence; middle trace, non-specific light scattering; bottom trace, cytochrome *a* absorbancy changes.

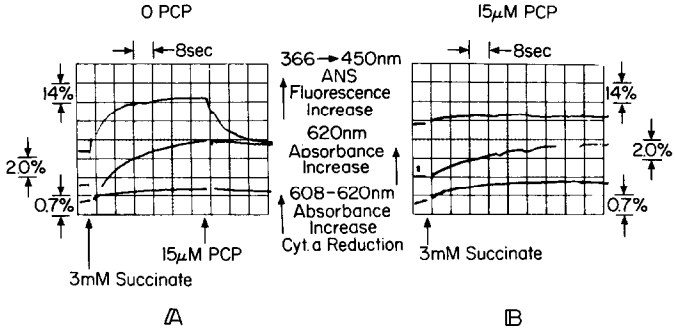

Fig. 3. Effect of permeant anions upon the response to an uncoupler. Experimental conditions were as in fig. 1B; others are as indicated.

adding succinate the ANS response is practically unchanged, but the NSLS response is greatly reduced. Upon addition of oligomycin, no further change in NSLS occurs, but the ANS fluorescence increases very rapidly, as in the previous experiment. It is apparent that here there is a qualitative difference in the response; the NSLS change is not associated with energy coupling induced by oligomycin.

3.2. *Effect of uncouplers on NSLS and ANS fluorescence*

Fig. 3A shows typical responses of NSLS and ANS; PCP is then added, causing the ANS fluorescence to decrease (half-time 6 sec). The response of NSLS is not detectable initially, and after 8 sec a slight decrease is observed. The response of an initially uncoupled system is shown in fig. 3B; 15 μM PCP is added prior to the initiation of electron transport by succinate. A scarcely detectable fluorescence change is observed in the ANS trace. However, NSLS proceeds, albeit with somewhat reduced velocity, to a level which approaches (after the end of the chart) the magnitude indicated in fig. 3A. It is apparent that NSLS is not affected by uncoupling agents to the same extent as the energy-dependent reaction causing the ANS fluorescence changes.

4. Discussion

The non-specific light-scattering change observable on activation of electron transport and energy coupling in membrane fragments derived from beef heart occurs very nearly simultaneously with the activation

of energy coupling, the latter indicated by the membrane-bound fluorochrome, ANS [6]. The two phenomena are resolvable upon activation of electron transport and energy coupling under two conditions. First, when electron transport and energy coupling are activated in the presence of permeant anions such as nitrate (and thiocyanate and iodide as well) employed at concentrations below those which diminish ANS fluorescence [7], nearly complete elimination of the NSLS changes is observed. Secondly, activation of electron transport in the absence of energy coupling shows significant NSLS changes which are not further increased when energy coupling is activated by the addition of oligomycin, ANS showing a large response at this time. Thus the NSLS change is not essential to either the simultaneous activation of electron transport and energy coupling upon addition of succinate or to the activation of energy coupling alone by oligomycin addition.

Experiments with uncouplers also give a definitive separation of the two effects; whereas the ANS fluorescence increase is rapidly diminished on addition of uncouplers, the decrease of NSLS during this interval is scarcely detectable. Further and most significant is the observation that activation of electron transport in PCP-uncoupled ESMP leads to a readily measurable NSLS increase and to a negligible increase of ANS fluorescence.

The conformational change reported by light-scattering in submitochondrial particles is dependent upon electron transport but is not related to recognizable energy-coupling events, either as a conformational state related to primary events of energy coupling or as a consequence of subsequent energy-dependent ion movements [17]. The structure or charge reorganizations involved in the energy-coupling reaction as reported by ANS may not show NSLS changes and thus may not be associated with the gross structure of the membrane, but instead with the molecular properties of the membrane components. The results further suggest that other probes for membrane structure (spin label, ORD, NMR, etc. [18]) must be examined for their relevance to energy coupling by appropriate kinetic and inhibitor-activator studies such as those described here.

References

[1] C.R.Hackenbrock, J. Cell. Biol. 30 (1966) 269.
[2] B.Chance and G.R.Williams, Adv. Enzymol. 17 (1965) 65.
[3] B.Chance and L.Packer, Biochem. J. 68 (1958) 295.
[4] C.R.Hackenbrock, J. Cell. Biol. 37 (1968) 345.
[5] G.Weber and D.J.R.Lawrence, Biochem. J. 56 (1954) 31P.
[6] A.Azzi, B.Chance, G.K.Radda and C.P.Lee, Proc. Natl. Acad. Sci. U.S. 62 (1969) 612.
[7] B.Chance, C.P.Lee and A.Azzi, Biochemistry, to be published.
[8] L.Packer and A.L.Tappel, J. Biol. Chem. 235 (1960) 525.
[9] cf. Discussion, in: Mitochondrial Structure and Compartmentation (Second Bari Round-Table) eds. E.Quagliariello, S.Papa, E.C.Slater and J.M.Tager (Adritica Editrice, Bari, 1967) p. 327.
[10] C.P.Lee and L.Ernster, in: Regulation of Metabolic Processes in Mitochondria (First Bari Round-Table), eds. J.M.Tager, S.Papa, E.Quagliariello and E.C.Slater (BBA Library Vol. 7, Elsevier, Amsterdam, 1966) p. 218.
[11] E.Racker, D.D.Tyler, R.W.Estabrook, T.E.Conover, D.F. Parsons and B.Chance, in: Oxidases and Related Redox Systems, eds. T.E.King, H.S.Mason and M.Morrison (Wiley & Sons, Inc., New York, 1965) p. 1077.
[12] L.Packer, M.P.Donovan, and J.M.Wrigglesworth, in: Probes for Membrane Structure and Function (Fourth Johnson Foundation Colloquium) eds. B.Chance, C.P. Lee and T.Yonetani (Academic Press, Inc., New York) in press.
[13] C.P.Lee and L.Ernster, Methods in enzymol. X (1967) 543.
[14] H.Tedeschi, J.M.James and W.Anthony, J. Cell Biol. 18 (1963) 503.
[15] R.Freedman and G.K.Radda, in: Probes for Membrane Structure and Function (Fourth Johnson Foundation Colloquium) eds. B.Chance, C.P.Lee and T.Yonetani (Academic Press, Inc., New York) in press.
[16] A.Azzi and H.Vainio, ibid.
[17] B.Chance, C.P.Lee and L.Mela, Fed. Proc. 26 (1967) 1341.
[18] Probes for membrane Structure and Function (Fourth Johnson Foundation Colloquium) eds. B.Chance, C.P.Lee and T.Yonetani (Academic Press, Inc., New York) in press.