FLUORESCEIN CONJUGATES OF CYTOCHROME *c* AS INTERNAL pH PROBES IN SUBMITOCHONDRIAL PARTICLES

John A. Thomas and Douglas L. Johnson*

Department of Biochemistry
The University of South Dakota School of Medicine
Vermillion, South Dakota 57069

Received June 9,1975

Summary: Conjugation of pure cytochrome *c* with fluorescein isothiocyanate produces derivatives whose fluorescence intensity increases approximately 40 per cent between pH 6 and 7. Preparation of sonic particles from cytochrome *c* - depleted mitochondria in the presence of the conjugates results in their incorporation behind the membrane barrier, as determined by inaccessibility to removal by KCl washes or to oxidation by ferricyanide. Energization of the sonic particles with substrates or oxygen causes an acidification of the environment of the probe, which is increased in the presence of valinomycin plus potassium, and abolished by nigericin or uncouplers.

INTRODUCTION

There is considerable evidence that proton movements are associated with the mechanism of oxidative phosphorylation in mitochondrial membranes. Despite much effort, it remains undetermined whether these proton movements represent the primary step of energy conservation as proposed by Mitchell (1), or are only a secondary process. Part of the difficulty has been the absence of an unequivocal kinetic method for monitoring proton movements in the space enclosed by the mitochondrial membrane.

Mitchell and Moyle (2) reported that submitochondrial particles prepared by sonication transport hydrogen ions inward, rather than outward, as seen with intact mitochondria. This is consistent with other evidence that the membranes of sonic particles are inverted with respect to the mitochondrial membrane (3, 4). Cytochrome *c*, for example, is easily removed from swollen mitochondria by isotonic KCl (5), while the same procedure fails for EDTA-submitochondrial particles, where the cytochrome is apparently behind the membrane permeability bar-

*Present address: Medical Incorporated, 5200 West 78th Street, Minneapolis, Minnesota 55435.

rier (6). This paper reports a method suitable for monitoring both the kinetics and magnitude of internal pH responses in submitochondrial particles by taking advantage of the membrane inversion upon sonication.

METHODS AND MATERIALS

Preparation of fluorescein derivatives of cytochrome *c*. pH-sensitive fluorescent derivatives of cytochrome *c* (Sigma, type VI) were prepared by reaction with a 5:1 ratio (w:w) of fluorescein isothiocyanate (Sigma, isomer I) for four hours at pH 7.8. The unreacted fluorescein was separated from the cytochrome by passage through a column of Sephadex G-25 (10 volumes). Several 1:1 derivatives of fluorescein:cytochrome *c* were purified by chromatography on Biorex 70, using a linear salt gradient to elute the derivatives. The complete preparative procedure and characterization of these derivatives will be published elsewhere.

Preparation of mitochondria and submitochondrial particles. Heavy beef heart mitochondria were prepared according to the method of Löw and Vallin (7), using a Brinkmann Polytron to homogenize the heart muscle mince. Mitochondria prepared by this method were found to be depleted in cytochrome *c* (see Figure 3B). The preparation of the sonic particles enriched in the cytochrome derivatives was essentially according to the method of Huang and Lee (8). The resulting submitochondrial particles were recentrifuged twice in 0.15 M KCl and once in 10 mM tris acetate-290 mM sucrose to remove the excess cytochrome derivative and salt, respectively.

RESULTS

Fluorescein-cytochrome *c* derivatives.

Since fluorescein isothiocyanate reacts with primary amine groups, there are 19 singly modified derivatives possible from horse heart cytochrome *c*. Fortunately, two lysines appear to be much more reactive with this reagent than the others, and these derivatives predominate in reaction mixtures having an average modification ratio of less than 1.5 moles fluorescein/mole cytochrome. These two derivatives have been purified on BioRex 70. Each has a fluorescein:cytochrome *c* ratio of 1.0:1, based on the extinction coefficient of 74,000 at 494 nm for fluorescent conjugates (9) and the spectrum of unmodified cytochrome *c* (10). Both of these derivatives show one major band on polyacrylamide gel electrophoresis, are enzymatically reducible, and are not auto-oxidizable (not shown). The absorption spectrum of one of these derivatives appears in Figure 1, both in oxidized and reduced form, and is essentially identical to the other derivative.

The fluorescence of these derivatives is not appreciably sensitive to ionic strength or solvent polarity, but is to pH (Figure 2). The fluorescent responses

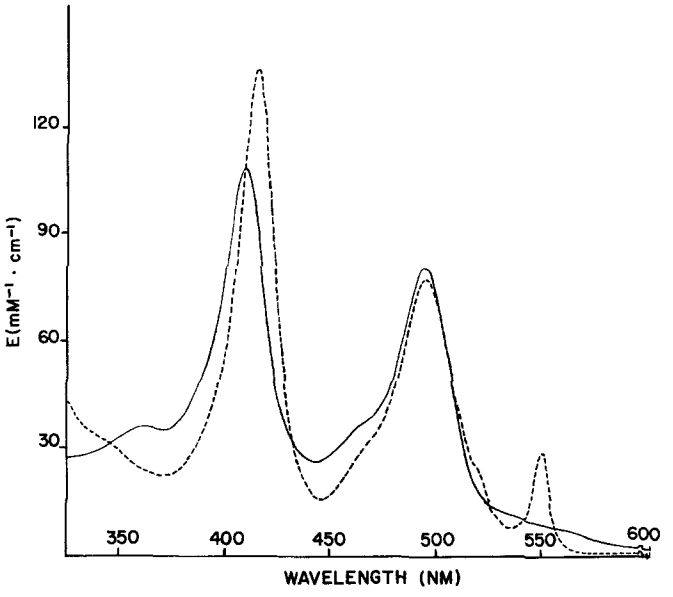

Figure 1. Oxidized and reduced spectra of a 1:1 fluorescein:cytochrome *c* derivative. Spectra of the derivative at 7.8 μM concentration in 0.1 M sodium phosphate, pH 7.8, in the presence of 5 μM potassium ferricyanide (———); in the presence of 5 mM sodium ascorbate (-----). The spectra were measured on a Beckman DB-G spectrophotometer. The extinction coefficients are based on a value of 74,000 for fluorescein conjugates (9) and 6,300 for cytochrome *c* (10) at 494 nm.

---

are reversible above pH = 5, with the fluorescent chromophore having a pK of about 6.3.

Figure 3A shows a typical spectrum (dithionite reduced minus oxidized) of submitochondrial particles in which the derivative has been incorporated. The cytochrome *c* absorption band at 550 nm is seen to dominate the spectrum. Cytochrome *c* to heme *a* ratios of 2 to 4 have been achieved by this procedure. Figure 3B shows the spectrum resulting when the same procedure was carried out omitting the addition of the cytochrome *c* derivative before sonication. In this case, only a shoulder is seen near 550 nm, which can be attributed mainly to cytochrome c_1.

It has been reported (8, 11) that partially inhibitory concentrations of KCN added to EDTA-submitochondrial particles supplemented with oligomycin and succinate result in biphasic reduction kinetics of cytochrome *c*. The slow phase can be abol-

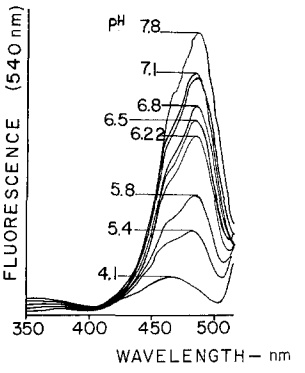

Figure 2. pH dependence of the fluorescence excitation spectra of a 1:1 fluorescein: cytochrome *c* derivative. The derivative at 7 μM concentration was initially in 0.02 M sodium phosphate buffer, pH 7.8. pH changes were accomplished by additions of N HCl. Spectra were measured on an Aminco Bowman SPF equipped with a thermostatted cell compartment, with the emission monochromator at 530 nm.

ished by the addition of FCCP*. These particles also exhibited this behavior (Figure 4), indicating that the cytochrome derivative functions normally in the electron transport chain. In addition, once the cytochrome was fully reduced, ferricyanide addition caused only about 15 per cent of the total amount of cytochrome *c* present to be oxidized (Figure 4). Cytochrome *c* in mitochondria under the same conditions (11), or added externally to these particles, is virtually 100 per cent oxidized by ferricyanide. This indicates that the cytochrome is indeed behind the membrane barrier in these particles.

These particles exhibited the energy-dependent fluorescence responses with ANS* typical of submitochondrial particles (Figure 5A) and opposite in direction to those seen with intact mitochondria (12). Succinate induced a small response, which was greatly enhanced by the addition of oligomycin, which is required for maximal coupling in these particles (3). Ascorbate plus phenazine methosulfate elicited a smaller response. In Figure 5B, the same sequence of additions was repeated in a separate experiment (minus ANS) only the fluorometer was set to mon-

*Abbreviations: FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; ANS, 8-anilino-1-naphthalene sulfonate.

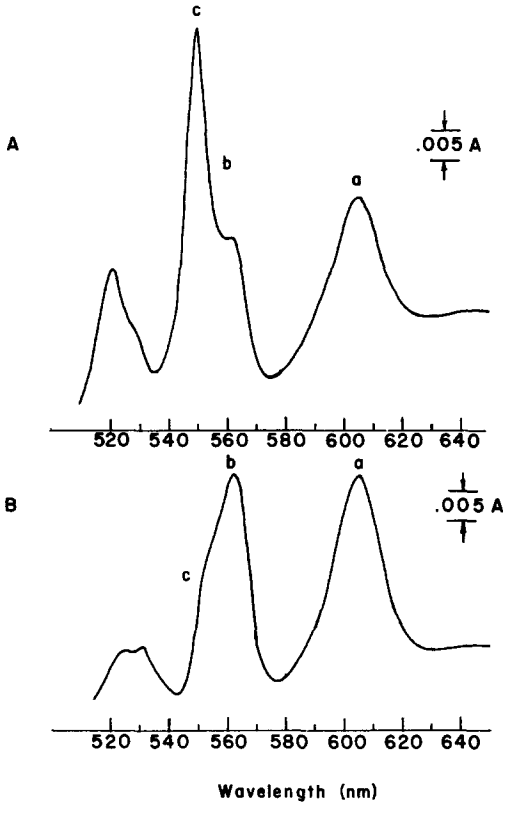

Figure 3. Difference spectra of submitochondrial particles prepared in the presence (3A) and absence (3B) of the fluorescein:cytochrome *c* derivative. Both reference and sample cuvettes contained submitochondrial particles at 1 mg/ml in 0.02 M sodium phosphate buffer, pH 7.8, plus 0.4 per cent sodium deoxycholate. Difference spectra were recorded on a Beckman DB-G with a scale expansion accessory after the addition of solid sodium dithionite to the sample cuvette.

---

itor the fluorescein responses (490 → 560 nm). Energization in this case induced a small but reproducible decrease in fluorescence, which is indicative of an acidification in the environment of the probe. This is the expected direction for an internally located probe in submitochondrial particles, according to the chemiosmotic coupling theory. FCCP removed all the energy-dependent responses, although succinate gave a small response, regardless of the presence of uncoupler, due to the succinate being at a higher pH than the suspending buffer. Calibration of the responses, obtained by adding aliquots of acid and base at the end of the experi-

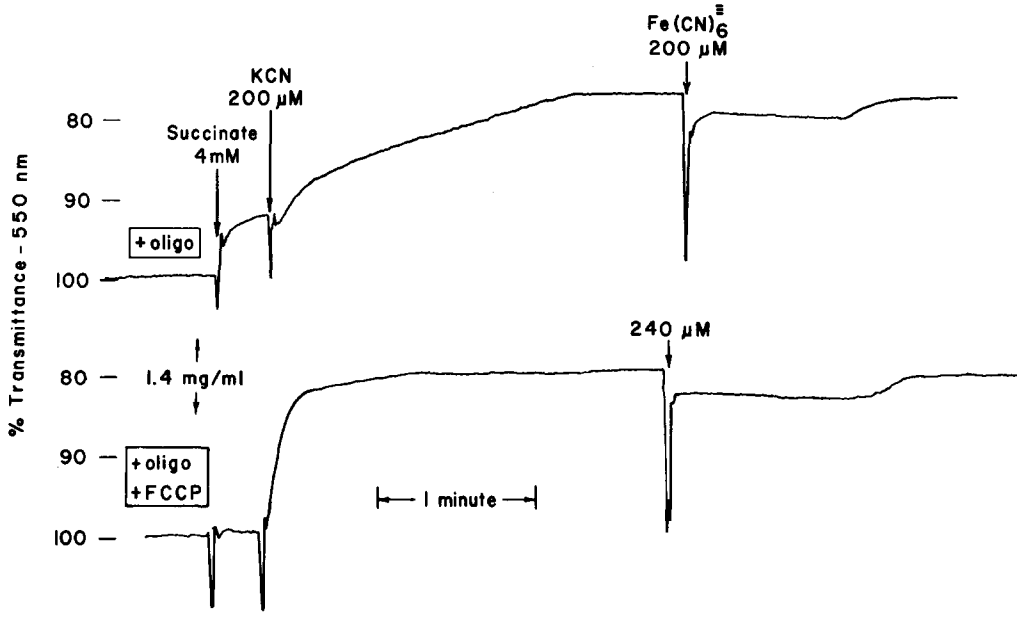

Figure 4. Reduction kinetics and ferricyanide accessibility of the cytochrome *c* derivative in coupled and uncoupled submitochondrial particles. Both reference and sample cuvettes contained submitochondrial particles at 1.4 mg/ml in 270 mM sucrose-50 mM tris acetate, pH 7.8, plus 1.7 μg oligomycin/ml. 1 μM FCCP was present in addition in the lower curve. Other additions as indicated.

---

ment, indicated that the internal pH change was less than 0.2 pH units under these conditions.

The magnitude of these responses was larger when the buffer was omitted, and particularly if KCl plus valinomycin were present initially. In Figure 6A, anaerobic-aerobic transitions in an unbuffered suspension of the submitochondrial particles (containing 13 mM succinate and 30 mM KCl) were obtained by the addition of hydrogen peroxide in the presence of catalase. The transmembrane pH gradient in the energized state was initially 0.4 pH, but could no longer be generated after the addition of nigericin. In Figure 6B, the same experiment was repeated in the presence of valinomycin. In this case, the pH gradient was initially about 0.8 pH, and was again dissipated by nigericin. Responses indicating a transmembrane pH gradient of over 1 pH unit have been observed in these particles when NADH was used to energize the membranes in the presence of valinomycin and KCl (not shown).

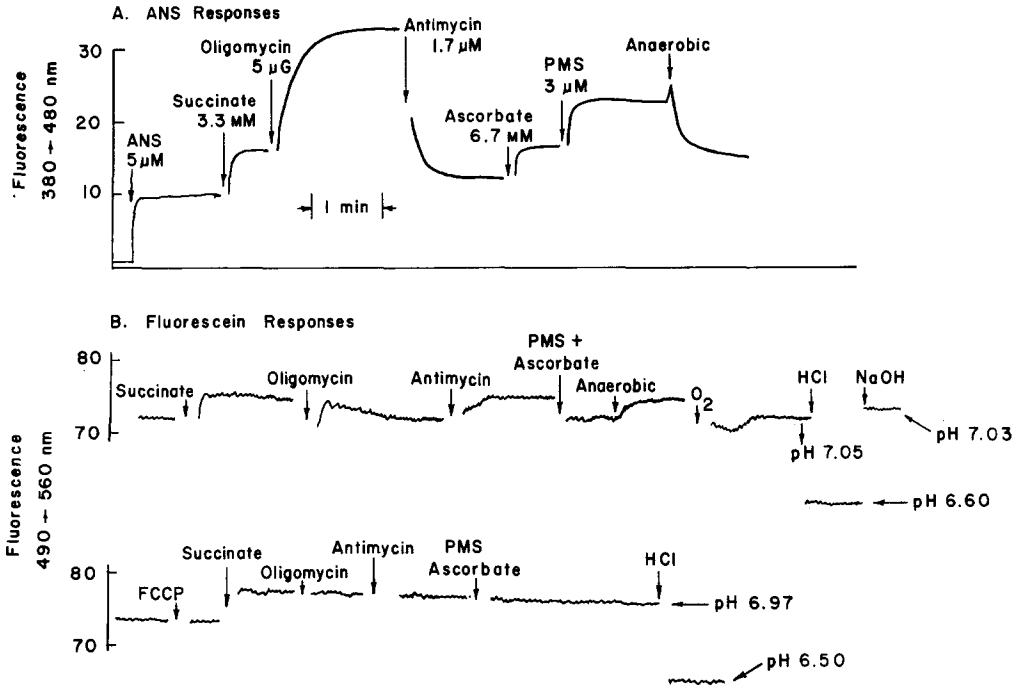

Figure 5. Energy-dependent fluorescence responses of (A) ANS and (B) fluorescein-cytochrome *c* in the fluorescein-cytochrome *c* submitochondrial particles. The reaction mixture consisted of 250 mM sucrose, 10 mM tris acetate, pH 7.0. In A, the submitochondrial particle concentration was 0.4 mg/ml; in B, 0.8 mg/ml. Other additions as indicated. The fluorometer was equipped with Glan polarizers on both the excitation and emission monochromators to minimize scattering artifacts (15).

DISCUSSION

The energy-dependent pH responses seen with these derivatives can all be interpreted in terms of the chemiosmotic theory of Mitchell. Energization causes an internal acidification in submitochondrial particles. Nigericin, which promotes a neutral exchange of H^+ for K^+, increases the membrane potential at the expense of the pH gradient, while valinomycin, which permits internal K^+ to diffuse out (down the electrical gradient), results in an increased pH gradient (13). The presence of both antibiotics or of the uncoupler FCCP uncouples these particles (13), and thus abolishes the internal pH responses.

The exact location of the fluorescein-cytochrome *c* derivative behind the membrane barrier is unknown. These experiments do not show whether the acidification

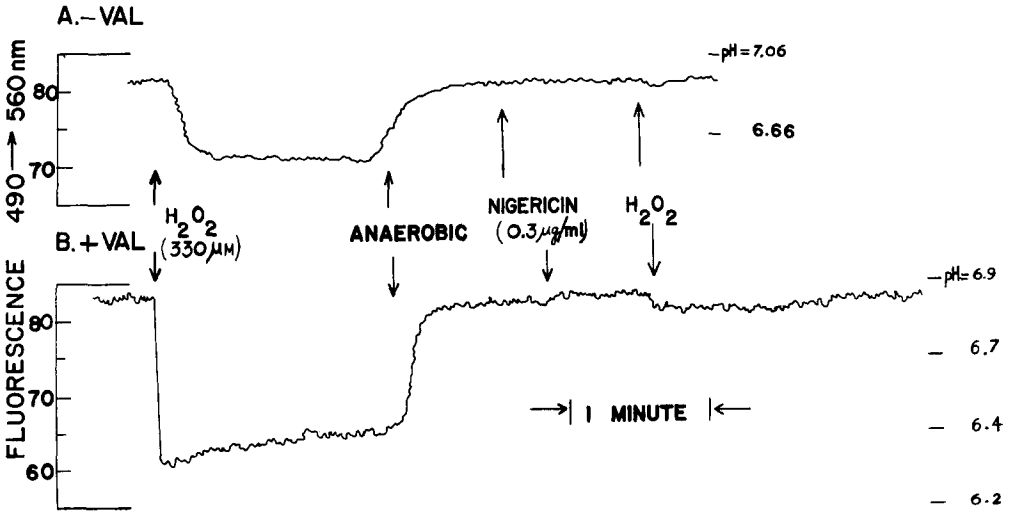

Figure 6. Energy-dependent fluorescence responses of fluorescein-cytochrome *c* in the fluorescein-cytochrome *c* submitochondrial particles in the (A) absence and (B) presence of valinomycin. The reaction mixture contained initially 0.55 mg/ml submitochondrial particles in 30 mM KCl, 230 mM sucrose, 0.2 μM catalase, 1.7 μg/ml oligomycin, and 7 mM sodium succinate, pH 7.0. Additions were made as indicated after the solution became anaerobic. Fluorometer settings identical to Figure 5.

responses occur within the membrane boundaries, or in the enclosed water space of these vesicles. Since cytochrome *c* binds at the surface of the inner membrane in mitochondria, the simplest interpretation is that it is also at the membrane-water interface on the interior of the inverted submitochondrial particles.

There is evidence that submitochondrial particles are not a homogeneous population of inverted vesicles, but rather a mixture of inverted and non-inverted vesicles (8, 11, 14). The responses presented in this paper would selectively come from the inverted vesicles, since the KCl washes remove the external cytochrome *c* from the non-inverted vesicles. Hence, only the cytochrome *c* located behind the membrane permeability barrier is left to monitor the pH responses. Simultaneous measurements with a pH electrode indicate the external pH responses are opposite in direction to those indicated by the fluorescent probe upon energization.

The method presented here for monitoring internal pH responses in submitochondrial particles offers a potential way for subjecting the chemiosmotic coupling

theory to more definitive testing than has been accomplished previously. Experiments are in progress to determine whether the kinetics of the internal pH responses correspond to the primary events in energy conservation.

Acknowledgments: This work was supported by Grant 71-SD-515 from the South Dakota Heart Association and a grant from The University of South Dakota School of Medicine Fund for Heart Research.

REFERENCES

1. Mitchell, P. (1961) Nature 191: 144-148.
2. Mitchell, P., and Moyle, J. (1965) Nature 208: 1205-1206.
3. Lee, C.P., and Ernster, L. (1966) in Regulation of Metabolic Processes in Mitochondria, eds. J.M. Tager, S. Papa, E. Quagliariello, and E.C. Slater, Elsevier Publishing Company, Amsterdam, pp. 218-236.
4. van Dam, K., and Meyer, A.J. (1971) Ann. Rev. Biochem. 40: 115-160.
5. Jacobs, E.E., and Sanadi, D.R. (1957) J. Biol. Chem. 235: 531-534.
6. Lee, C.P. (1971) in Probes of Structure and Function of Macromolecules and Membranes, eds. B. Chance, C.P. Lee, and J.K. Blaisie, Vol. I, Academic Press, New York, pp. 417-426.
7. Löw, H., and Vallin, I. (1963) Biochim. Biophys. Acta 69: 361-374.
8. Huang, C.H., and Lee, C.P. (1975) Biochim. Biophys. Acta 376: 398-414.
9. Brusman, H. (1971) Anal. Biochem. 44: 606-611.
10. Margoliash, E. (1959) Biochem. J. 71: 559-570.
11. Chance, B., Erecinska, M., and Lee, C.P. (1970) Proc. Nat. Acad. Sci. 66: 928-935.
12. Azzi, A. (1969) Biochem. Biophys. Res. Commun. 37: 254-260.
13. Montal, M., Chance, B., and Lee, C.P. (1970) J. Membrane Biol. 2: 201-234.
14. Astle, L., and Cooper, C. (1974) Biochemistry 13: 154-160.
15. Passwater, R.A. (1973) Fluorescence News 7: 17-23.