Biochimica et Biophysica Acta, 459 (1977) 216–224

BBA 47235

CATION TRANSPORT IN CYTOCHROME OXIDASE RECONSTITUTED VESICLES

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(Received October 12th, 1976)

SUMMARY

Cation translocation across the membrane of cytochrome oxidase reconstituted vesicles may be followed with a simple spectrophotometric method. Cytochrome oxidase reconstituted vesicles, supplemented with ascorbate and cytochrome *c*, induce large spectral changes of the positive dye safranine, reversed by uncouplers and inhibitors of respiration. The dye is probably accumulated in the inner space of the vesicles, where it reaches high concentrations and aggregates. The spectral shifts and the absorbance changes, due to aggregation, are proportional to the amount of the dye taken up and depend on the respiratory control. In the presence of potassium, valinomycin causes an inhibition, whereas nigericin stimulates the dye uptake.

The data are discussed in terms of electrical potential dependent fluxes.

INTRODUCTION

Incorporation of cytochrome oxidase into phospholipid vesicles reconstitutes a biologically active system which appears to be a simple and valuable tool for the study of respiratory control and ion transport. Although experiments concerning the respiration and the effect of uncouplers and ionophores on the oxygen consumption have been reported recently [1–7], the process of cation translocation has been discussed only by Hinkle [2, 3, 7]. In the presence of valinomycin, during respiration, the reconstituted vesicles accumulate potassium in exchange with hydrogen ions.

In the present paper we report a spectrophotometric method which permits us to investigate in more detail the mechanism of cation translocation. This method is based on the metachromatic properties of the positive dye safranine.

As the dye concentration increases, a deviation from the Beer-Lambert law is

Abbreviations used: FCCP: carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; Tris: Tris(hydroxymethyl)aminomethane; EDTA: disodiumethylenediaminetetraacetate; HEPES: *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

observed, due to formation of dye dimers and multimers in solution [8, 9]. The spectral changes induced by the dye aggregation consist in a decrease of the absorbance coefficient of the monomeric band at 520 nm, while another band appears at the shorter wavelengths, attributed to the multimeric form. The same spectral changes were shown by addition of a polyanion even at very low dye concentrations. In the latter case the interaction among the dye molecules is induced by the dye binding to nearby negative sites of the polyanion [8]. The dye accumulates into the inner space of the vesicles and gives large absorbance changes due to aggregation of the dye molecules. The dye accumulation is affected by: (i) the presence of permeant cations, (ii) the degree of the membrane permeability to cations, (iii) the cation gradient across the membrane and (iv) the nature of the cation ionophore. The cation translocation may be explained by the hypothesis that the vectorial electron transfer through the enzyme leads to hydrogen ion translocation and to a transmembrane electrical potential, negative inside.

MATERIALS AND METHODS

Cytochrome oxidase was prepared according to the method of Yonetani [10]. Suspensions of the enzyme in 0.1 M phosphate buffer and 1 % Tween 80 were frozen under liquid nitrogen and stored at −20 °C.

Lyophilized soybean phospholipids suspended in a medium containing 50 mM Tris-phosphate buffer, at pH 7.2, and 0.2 mM EDTA/Tris, were sonified with a Branson Sonifier, under a nitrogen atmosphere, for 10 min, in a jacketed vessel maintained at 2 °C by a constant-temperature circulating bath. Phosphorus (P_i) was determined by the method of Fiske and SubbaRow [11]. Reconstitution of cytochrome oxidase vesicles without dialysis was performed as described by Racker [1]: the vesicles (27 mM P_i) were mixed with 0.3 mg/ml of cytochrome oxidase in a medium containing 1 % cholate and 50 mM Tris/phosphate buffer, at pH 7.2. The mixture was maintained at 4 °C for 4–5 hours before the experiments. The amount of cytochrome oxidase vesicles is indicated henceforth in μg protein/ml. The lipid/oxidase ratio for each preparation was approximately 60 μg phospholipids/μg protein. Chromatography in a column of Sepharose 6B showed identical elution peaks for cytochrome oxidase reconstituted vesicles and control phospholipid vesicles without cytochrome oxidase. The binding of the enzyme to the vesicles therefore did not change the dimension of the vesicles.

Oxygen consumption was measured with a Clark electrode, at 20 °C. Absorbance spectra were detected in a Perkin Elmer spectrophotometer model 124. Absorbance changes of the safranine were detected in a non-commercial double-wavelength spectrophotometer at 530 nm, taking as reference wavelength 578 nm. Hydrogen ion changes were detected with a Radiometer PHM 26 pH meter connected with a Texas recorder.

Cholate was a product of Merck and purified according to the method of Hinkle et al. [2]. Soybean phospholipids (from Lucas Meyer, Hamburg) were purified according to the method of Kagawa and Racker [12]. Safranine was a product of BDH and was purified according to the method of Pal and Schubert [13]. Cytochrome *c*, type VI, was supplied by Sigma.

RESULTS

Interaction between safranine and cytochrome oxidase reconstituted vesicles

The spectrum of the dye in the presence of cytochrome oxidase reconstituted vesicles shows a shift of the λ_{max} of the monomeric peak from 520 to 530 nm. The same shift was observed by replacing H_2O with ethanol in pure dye solutions. The dye probably binds passively to the membrane vesicles in a partially apolar micro-environment.

Addition of ascorbate and cytochrome *c* induces dye aggregation, as indicated by the spectral changes of Fig. 1A. Uncouplers and inhibitors of the respiration restore the initial spectrum. Fig. 1B shows the kinetics of the dye absorbance changes in the presence of respiring vesicles and after addition of either FCCP or NaCN. Fig. 1C shows the effect of the dye addition to respiring cytochrome oxidase vesicles. An activation of the steady-state respiration and a concomitant release of H^+ was observed. However, some preparations lacked this stimulation. The dye/hydrogen ion stoicheiometry, assuming that all the dye added has been taken up, was 6–7. Correction was made for interference of the dye with a hydrogen-glass electrode.

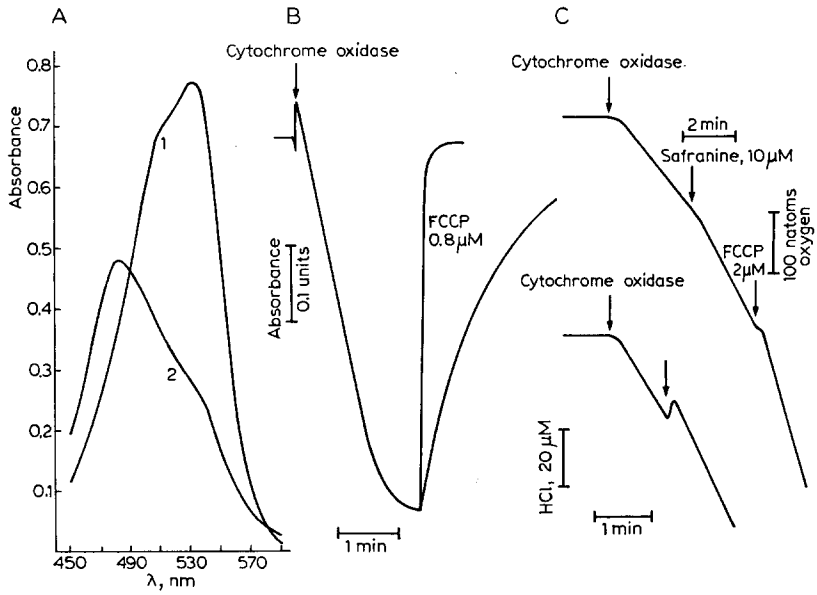

Fig. 1. Interaction between safranine and cytochrome oxidase reconstituted vesicles. (A) Spectrum of safranine in the presence of cytochrome oxidase vesicles (10 μg/ml) before (1) and after (2) addition of ascorbate and cytochrome *c*. The medium contained in a volume of 2.5 ml: 0.1 M sucrose, 10 mM Tris/Cl pH 7.2, 0.2 mM EDTA/Tris. Safranine was 30 μM, sodium ascorbate 50 mM and cytochrome *c* 25 μM. (B) Kinetics of the absorbance change during uptake and release of safranine. The reaction started with cytochrome oxidase vesicles (10 μg/ml). The medium and volume were the same as in A. Dual wavelengths measurements at 530–578 nm. (C) Oxygen consumption and proton movements induced by safranine. The oxygen consumption (upper part of the figure) started with cytochrome oxidase vesicles (6 μg/ml). The medium was the same as in A except for safranine. Final volume 2 ml. The proton movements (lower part of the figure) started with cytochrome oxidase vesicles (12.5 μg/ml). The medium contained in a volume of 4.0 ml: 0.1 M sucrose, 500 μM Tris/Cl pH 7.2, 0.2 mM EDTA/Tris, 10 mM Sodium ascorbate and 25 μM cytochrome *c*.

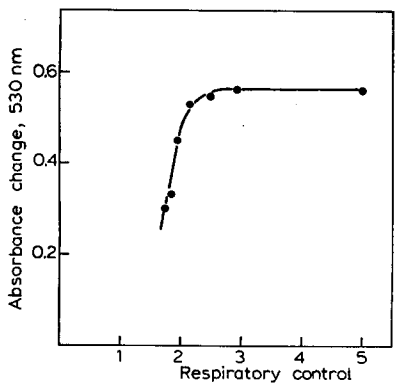

Fig. 2. Dependence of the absorbance change on the respiratory control. The medium and the volume were the same as in Fig. 1A. The respiratory control was the ratio between the rates of oxygen consumption after and before the addition of 0.25 μg/ml of valinomycin and nigericin. The absorbance change was measured in the presence of 0.2 μg/ml of nigericin. Each experimental point represents a single preparation. The amount of protein was approximately the same for each experiment.

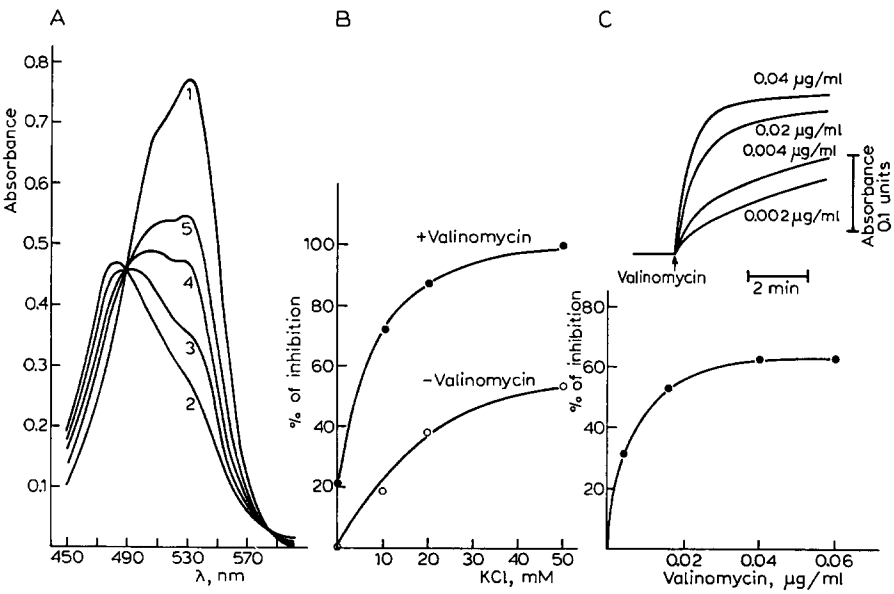

Fig. 3. Effect of KCl and valinomycin on the dye absorbance. (A) Dye spectra in the presence of various KCl concentrations. (1) 30 μM EDTA Tris and 10 μg/ml of cytochrome oxidase vesicles. Total volume 2.5 ml; (2) after addition of 50 mM Sodium ascorbate and 25 μM cytochrome *c*; (3) 0.1 M sucrose was replaced with 0.01 M KCl and 0.08 M sucrose; (4) 0.1 M sucrose was replaced with 0.02 M KCl and 0.06 M sucrose; (5) 0.1 M sucrose was replaced with 0.05 M KCl. (B) Percentage of inhibition of the absorbance change induced by KCl. The medium was the same as in A. The osmolarity was kept constant by adding variable amounts of sucrose. The percentage of inhibition was calculated at 530 nm as the ratio between the absorbance change induced by KCl, either in the absence or in the presence of valinomycin and the total absorbance decrease induced by Sodium ascorbate and cytochrome *c* in the absence of KCl. (C) Absorbance changes and percentage of inhibition at various valinomycin concentrations. The medium was the same as in A. 7 mM KCl was also present. Upper: valinomycin added after the dye uptake. Lower: valinomycin added before the dye uptake.

The dye aggregation takes place in a Ca^{2+}-inaccessible space, since the dye spectral shifts during respiration are not influenced by the presence of $CaCl_2$. The dye is probably accumulated in the inner space of the vesicles. The absorbance change is proportional to the amount of dye taken up assuming that no appreciable passive dye binding to the vesicles occurs during respiration and only dye dimers and monomers are present in the inner space of the vesicles.

Fig. 2 shows the dependence of the absorbance change on the respiratory control. Each point represents a different preparation. The dye uptake is proportional to the respiratory control from 1 to about 2.2, and then it reaches a plateau for higher respiratory control values. The plateau can be explained by assuming that all the dye has been transported under these conditions. In fact when the same experiment is carried out with a higher concentration of safranine, higher values of absorbance changes are obtained and the plateau is reached at higher respiratory control values. The proportionality between dye uptake and respiratory control indicates that the absorbance changes may be taken as a criterion of a good reconstitution.

Substituting in the reconstitution 50 mM Tris/phosphate with different media such as: 50 mM KCl and 10 mM HEPES, 50 mM Tris/Cl, 50 mM potassium phosphate, did not modify the dye uptake; however the dye uptake was higher when reconstitution was performed in Tris/phosphate buffer, the respiratory control being the same.

Effect of salts and valinomycin

Fig. 3A shows that the absorbance change decreases by increasing the external

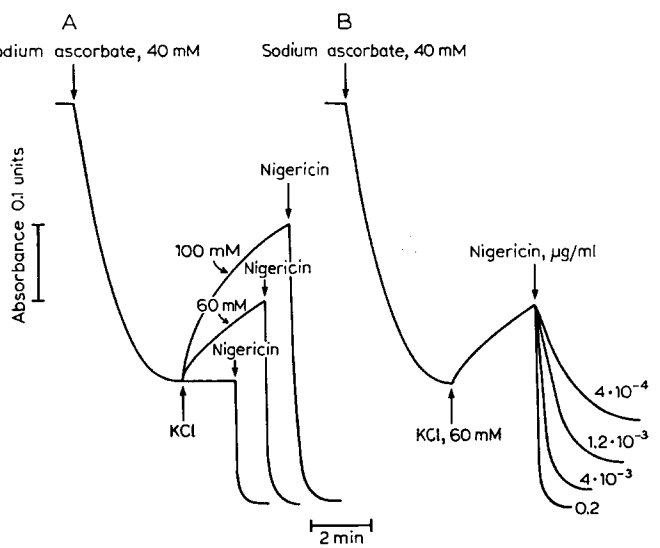

Fig. 4. Effect of KCl and nigericin on the dye absorbance (530 nm). The medium contained: 0.1 M sucrose, 10 mM Tris pH 7.2, 0.2 mM EDTA Tris, 20 μM cytochrome *c*, 30 μM Safranine and 6 μg/ml of cytochrome oxidase vesicles. Final volume 2.5 ml. (A) Absorbance changes due to nigericin (0.2 μg/ml) after addition of increasing KCl concentrations. (B) Absorbance changes due to increasing nigericin concentrations after addition of KCl.

KCl concentration. The following observations were made: (i) the extent of inhibition of the absorbance change depends on the nature of the cations, according to the sequence: $K^+ > Na^+ > Li^+ > Tris^+$; (ii) low concentrations of $CaCl_2$ (0.2 mM) counteract the salt inhibition; (iii) the respiratory control and the initial rate of oxygen consumption are not influenced by 50 mM KCl. A salt effect on the association of the cytochrome c with the membrane can therefore be excluded; (iv) reconstitutions in 100 mM KCl are less sensitive to the external KCl concentration, as a matter of fact 50 mM KCl causes an inhibition of about 20 %, while in vesicles reconstituted in Tris/phosphate the same external KCl causes an inhibition of 55 %; (v) addition of KCl after the dye uptake causes an absorbance increase, whose rate depends on the salt concentration, as shown later. No absorbance change was observed when KCl was replaced with sucrose. In the absence of added salt the absorbance returns slowly to the initial level, the ion in this case is Na^+, present as 40 mM Sodium ascorbate. Tris/ascorbate used instead of Sodium ascorbate maintains the equilibrium until anaerobiosis.

Valinomycin potentiates the inhibition of KCl, as shown in Fig. 3B. The extent and the rate of inhibition depend on the amount of ionophore (Fig. 3C). The dye uptake inhibition seems therefore to be dependent on the rate of penetration of the cations across the membrane, the extent of inhibition being dependent either on the cation gradient or on the permeability of the membrane to the cation. $CaCl_2$, at low concentration, probably modifies the permeability of the vesicles to the monovalent cations by changing the surface potential.

Effect of nigericin

Figs. 4A and B show that the addition of nigericin increases the dye absorbance change. The effect does not change if KCl was replaced with potassium phosphate or by adding Tris/Cl. The absorbance change increase therefore is due neither to a Cl^- penetration nor to a dye-Tris exchange. High amounts of nigericin cause a rapid absorbance decrease, and the final absorbance level is independent of the external

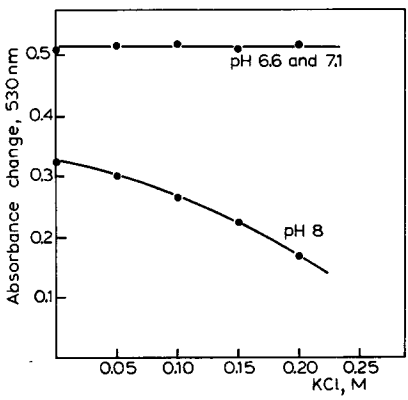

Fig. 5. Dependence of the absorbance changes from the external pH and KCl. The medium was the same as in Fig. 4, except for the external pH and KCl, as indicated in the figure. 0.2 μg/ml of nigericin was present.

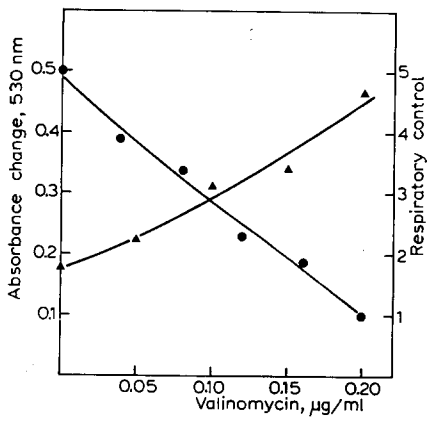

Fig. 6. Effect of nigericin and valinomycin on the absorbance changes and respiratory control. The medium contained: 0.1 M sucrose, 10 mM Tris pH 6.6, 20 mM KCl, 0.2 mM EDTA/Tris, 40 mM ascorbate/Tris, 20 μM cytochrome *c*, 30 μM Safranine, 0.28 μg/ml of nigericin and 9 μg/ml of cytochrome oxidase vesicles. The respiratory control was the ratio between the rates of oxygen consumption after and before the addition of nigericin and valinomycin. ●–●, absorbance changes; ▲–▲, respiratory control.

KCl concentration, as shown in Fig. 4A. Lower amounts of nigericin cause a slower absorbance decrease (Fig. 4B) and the final absorbance level depends on the nigericin/external KCl ratio. The effect shown by nigericin, as that shown by valinomycin, seems to be dependent on the rate of K^+ translocation across the membrane via ionophore.

Fig. 5 shows that, in the presence of nigericin the absorbance change is related either to the external KCl concentration or to the pH. The absorbance change is independent of the external KCl at acidic or neutral pH, while at alkaline pH, the increase of the external KCl concentration causes a decrease in the absorbance change. Therefore the prevailing force responsible for the K^+ movement via nigericin is a pH gradient. Internal alkalinization due to the respiration was indirectly measured by Hinkle [3].

Effect of nigericin plus valinomycin

The effect of ionophores seems to be of kinetic nature. The absorbance change should therefore be dependent on the nigericin/valinomycin ratio. Fig. 6 shows that, in the presence of high amount of nigericin, valinomycin causes an absorbance change inhibition whose magnitude depends on the amount of valinomycin. The respiratory control increases in parallel.

DISCUSSION

The spectrophotometric changes of the dye safranine represent a simple method to characterize the mechanism of cation translocation across the membrane of reconstituted cytochrome oxidase vesicles. The results obtained with this method show that: (i) the vesicles are able to translocate the cationic dye safranine, (ii) the amount of dye taken up is proportional to absorbance changes of the monomeric

band, (iii) the absorbance changes are proportional to respiratory control values, (iv) K^+ movements, mediated by valinomycin or nigericin, may be indirectly followed by the dye absorbance changes.

The hypothesis that respiration of cytochrome oxidase reconstituted vesicles causes an hydrogen ion translocation across the membrane and a transmembrane electrical potential, negative inside, accounts for all phenomena concerning the effect of uncouplers and ionophores on the oxygen consumption [1–3, 5–7], the kinetics of translocation of K^+ and H^+ [2, 3, 7], and lipophilic anions in inverted reconstitutions [14, 15]. The uptake of safranine and the effect of ionophores on the absorbance changes may be explained according to this hypothesis. The positive dye is accumulated and aggregates in the inner space as a consequence of the electrical potential. The penetration of an external permeant cation leads to a collapse of the electrical potential and to a decrease of the amount of dye taken up. The higher the permeability of the membrane to the cation (by addition of valinomycin in the case of K^+), the higher the inhibition by the cation. However, as occurs in mitochondria [16], in the final equilibrium state the amount of dye taken up should be independent of the presence of other permeant cationic species. As the respiration rate, in the presence of 50 mM external KCl is not dependent on the presence of valinomycin, one can exclude that the system is partially uncoupled. A first explanation may be that an external cation, exchanging with an hydrogen ion, further increases the inner pH of the vesicles. The electrical gradient therefore is partially converted in a pH gradient. Internal alkalinization due to respiration was shown by Racker [1] and by Hinkle [3]. A second explanation is that the vectorial electron transfer across the enzyme forms a large surface potential in the inner side of the cytochrome oxidase vesicles. The magnitude of this potential is controlled by the counterions by a mechanism either of screening or of binding [17]. In this case competition between different cations is possible.

Nigericin tends to increase the dye uptake either by reconverting the pH gradient in an electrical gradient or by maintaining the K^+ concentration in the inner space of the vesicles at low values.

A transmembrane oxidoreduction mechanism mediated by cytochrome oxidase leading to an electrical gradient, is the simplest and most reasonable explanation of the data reported in the present paper. However the question arises if this mechanism is still tenable in the whole mitochondrial system. Artificial systems for transmembrane electron transfer made by an oxidizing agent in the inner space, an external electron donor and an electron carrying mediator [3], are able to form an electrical gradient. Electron transfer through cytochrome oxidase reconstituted vesicles may represent therefore an oversimplification with respect to the more complex native mitochondrial system.

ACKNOWLEDGEMENTS

Professor Yonetani is gratefully thanked for introducing Dr. R. Colonna to the study and preparation of cytochrome oxidase during a short-term Nato Senior Fellowship at the Johnson Research Foundation, Philadelphia. The authors are grateful to Dr. P. Arslan for revising the English version of the paper and to Professor G. F. Azzone for his encouragement and criticism.

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