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Occurrence of a Reduced Nicotinamide Adenine Dinucleotide Oxidase Activity Linked to a Cytochrome System in Renal Brush Border Membranes†

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ABSTRACT: NADH oxidase activity has been detected in luminal membrane derived from the proximal tubules of rat kidney (brush border membranes). The cytochromes associated with these membranes [García, M. L., Benavides, J., Valdivieso, F., Mayor, F., & Giménez-Gallego, G. (1978) *Biochem. Biophys. Res. Commun. 82*, 738-744] were reduced by NADH, the extent of this reduction being affected by the presence of oxygen and by inhibitors of this NADH oxidase activity. These results suggest that the cytochromes act as an electron transport system between NADH and oxygen. The possibility of mitochondrial contamination is excluded on the basis of differential sensitivity to inhibitors and marker enzyme activities.

The existence of cytochromes in nonmitochondrial membranes of eukaryotic cells is well documented. Berezney et al. (1972) and Berezney & Crane (1972) have reported the presence of cytochromes in the nuclear membrane, and there is considerable information about microsomal cytochromes. NADH dehydrogenase activities have also been described in a wide variety of plasma cell membranes [for an excellent review, see Crane et al. (1979)]. In some cases it has been reported that these plasma membrane NADH dehydrogenases can utilize oxygen as a terminal electron acceptor (Gayda et al., 1977).

In a previous paper (García et al., 1978) we have reported the existence of cytochromes in a specialized plasma membrane, the luminal membrane derived from the proximal tubules of rat kidney (brush border membranes). These cytochromes could only be partially reduced by NADH, and the preparation did not show appreciable rates of NADH-dependent oxygen reduction. Several modifications of the procedure used to prepare a brush border membrane fraction with

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a stable NADH oxidase activity are reported in this paper. The properties of this activity have been studied. The results suggest that the cytochromes present in the brush border membranes may participate in the electron transport from NADH to oxygen. The oxidase activity appears to be intrinsic to the brush border preparation and not due to a microsomal or mitochondrial contamination.

Experimental Section

Methods

Isolation of Brush Border Membranes. Adult rats of the Wistar strain weighing 150–200 g were used. Luminal membranes derived from the proximal tubules were isolated by the method of Thuneberg & Rostgaard (1968) as modified by Aronson & Sacktor (1974) and Mitchell et al. (1974). The isolation medium was previously filtered through Millipore filters (0.22 μm) in order to avoid possible bacterial contamination.

Membranes prepared in 0.5 M sucrose were suspended in a medium containing 300 mM mannitol and 20 mM Hepes–Tris[1] (pH 7.4) and centrifuged at 27000*g* for 5 min. The pellet was washed 3 times with the same medium by resuspension and centrifugation at 12000*g* for 5 min. The final pellet was resuspended in the medium, frozen in 0.5-mL samples in liquid N_2, and kept at −80 °C. The activities studied were not substantially modified after several weeks of storage.

Isolation of Mitochondria. Mitochondria from the renal cortex were prepared by the method of Schneider (1948). The isolation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM Tris-HCl, and 1 mM EGTA, pH 7.4. After centrifugation at 800*g* for 10 min, mitochondria were centrifuged from the supernatant at 8200*g* for 10 min. The pellet was resuspended in 0.33 M sucrose and centrifuged at 8200*g* for 10 min. The final pellet was resuspended in the same medium used for the brush border membranes, frozen in 0.5-mL samples in liquid N_2, and kept at −80 °C.

Isolation of Microsomes. The cortex kidney microsomal fraction was obtained essentially as described by Baginsky et al. (1974). After centrifugation at 16000*g* for 20 min, the supernatant obtained was centrifuged at 105000*g* for 1 h. The final pellet was resuspended in the same medium used with the brush border membrane fraction.

Determination of Enzyme Activities. Membrane marker enzymes were assayed as follows: alkaline phosphatase by the method of Walter & Schutt (1974); succinate dehydrogenase according to Porteous & Clark (1965); glucose 6-phosphatase as indicated by Baginsky et al. (1974); glutamate dehydrogenase by the method of Schmidt (1974); oligomycin-sensitive ATPase according to Soper & Pedersen (1976).

NADH oxidase activity was measured by following optically the decrease in NADH concentration at 30 °C in a Bausch and Lomb Spectronic 710 connected to a recorder through a digital-analogic converted.

For an estimation of the K_m for NADH and V_{max} of the NADH oxidase system, complete oxidation of the substrate was followed in a Perkin-Elmer 356 spectrophotometer. The calculations were performed as described by Valle-Tascón et al. (1977).

Oxygen uptake was measured in a 2-mL thermostatic cuvette at 30 °C with a Clark-type electrode, connected to a Hitachi Model 1002 recorder.

Cytochrome Measurement. The spectra of cytochromes were recorded at room temperature with a Perkin-Elmer 356 spectrophotometer interfaced to a Tracor Northern digital signal averager, NS 570. Subtraction of base line and calculations of cytochrome content were performed from the digital data stored in the averager.

Protein Determination. Protein content of the samples was estimated according to the method of Lowry et al. (1951) with crystalline bovine serum albumin as standard.

Results

NADH Oxidase Activity of the Brush Border Membrane Preparation. In the standard brush border membrane preparation used in our previous studies on the brush border cytochromes, no NADH oxidase activity was detected (Garcia et al., 1978). However, a NADH-dependent oxygen reduction appeared after the isosmotic substitution of KCl from the suspension medium by mannitol and the adoption of several changes in the storage procedure [see Methods and García et al. (1978)]. No NADH oxidation was detected when the reaction mixture was made anaerobic.

Several enzymatic activities have been measured to evaluate the purity of the preparation in order to check if the origin of this NADH oxidase activity was a decrease of the purity of the brush border fraction due to any of the modifications. From Figure 1, assuming that succinate dehydrogenase activity is exclusively mitochondrial (Schneider, 1959), it can be estimated that mitochondria cannot account for more than 8% of the brush border preparation protein. Similar values for mitochondrial contamination can be estimated from the levels of oligomycin-sensitive ATPase of the preparation (Figure 1) and from glutamate dehydrogenase activity (results not shown), the level of which appeared equivalent to those previously reported for the brush border preparations (García et al., 1978). Na,K-ATPase activity was enriched 4 times with respect to the initial homogenate during the brush border preparation. However, the controversy on the basolateral or brush border membrane localization of this activity (Sacktor, 1977) disauthorizes the employment of this activity as a marker enzyme. Important microsomal contamination can also be discarded since the glucose-6-phosphatase activity of the preparation was almost negligible (<10% of that of homogenate) (results not shown). Finally, the brush border fraction appears enriched in alkaline phosphatase activity (Figure 1). The extent of this enrichment is in agreement with that previously reported by Berger & Sacktor (1970) for the brush border membranes. It must be mentioned that the enrichment in NADH oxidase activity of the brush border fraction is not as high as that found for alkaline phosphatase (Figure 1). This behavior might be expected from an enzyme nonspecifically localized in the brush border fraction. The enrichment in the NADH oxidase specific activity is of the same order as that reported for other activities nonexclusively associated to this membrane fraction, such as cyclic nucleotide phosphodiesterase (Filburn & Sacktor, 1976) and HCO_3^--stimulated ATPase (Liang & Sacktor, 1976).

Characterization of the Brush Border NADH Oxidase Activity. The brush border NADH oxidase activity has been assayed by following spectrophotometrically the disappearance of NADH from the assay mixture (see Methods). Equivalent results were obtained when the measurement was carried out by following polarographically the decrease of oxygen concentration upon the addition of NADH.

The brush border NADH oxidase presented a very stable activity at neutral pH. The reaction rate remained unchanged,

[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

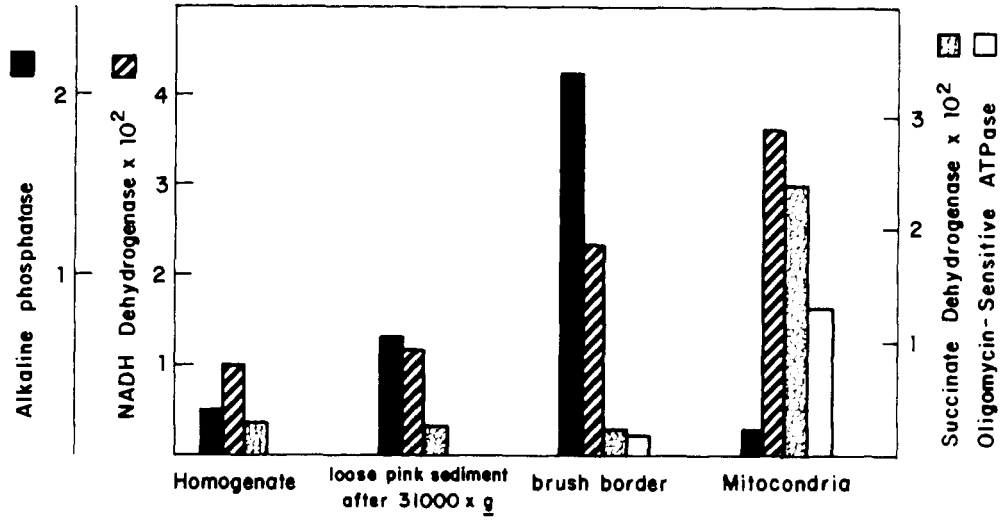

FIGURE 1: Specific activities of NADH oxidase and marker enzymes in several fractions of rat kidney. Enzyme determinations were carried out as indicated under Methods. NADH oxidase was assayed in 2 mL of 260 mM mannitol, 20 mM KCl, 3 mM Hepes–Tris (pH 7.4) buffer, and 100 μM NADH. The reaction was started by addition of 0.2 mg/mL freeze-thawed membrane suspension protein. The specific activities are expressed in micromoles per minute per milligram of protein.

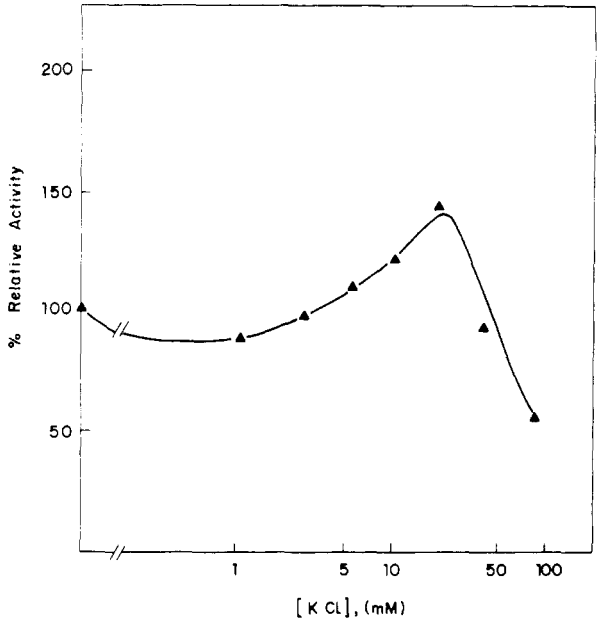

FIGURE 2: Effect of KCl on the brush border membrane NADH oxidase activity. For experimental details, see Figure 1 caption. KCl was omitted from the reaction mixture and added at variable concentrations.

at least for 1 h, under the assay conditions, provided that NADH was supplied. This stability was not due to the modifications introduced in the storage procedure. However, at lower pH values, even the initial rate was higher; the activity was not linear, rather it showed a decrease with time. Brush border NADH oxidase activity appeared to be modulated by KCl and NaCl. Figure 2 shows a plot of the effect of KCl concentration on this activity. NaCl also affected the activity, although the maximum stimulation reached only 50% of that elicited by KCl and occurred at 65 mM (results not shown). Similar results were obtained both with KCl and NaCl when the osmolarity of the reaction mixture was kept constant with sucrose.

As observed in Figure 3a, NADH oxidase activity was inhibited by NADH concentrations above 0.2 mM. A similar inhibition has already been described for mitochondrial NADH oxidase and was attributed to the nonlinear response of the spectrophotometer at high NADH concentrations (Singer, 1974). In our experiments this effect could not be attributed to the measurement system because the rate of NADH oxidation was not changed when the measuring beam was dimmed in order to mimic the optical effect of high nucleotide levels. NAD^+ up to 0.5 mM did not affect the reaction rate independent of the NADH concentration; thus, inhibition does not appear to depend on the redox state of added NAD.

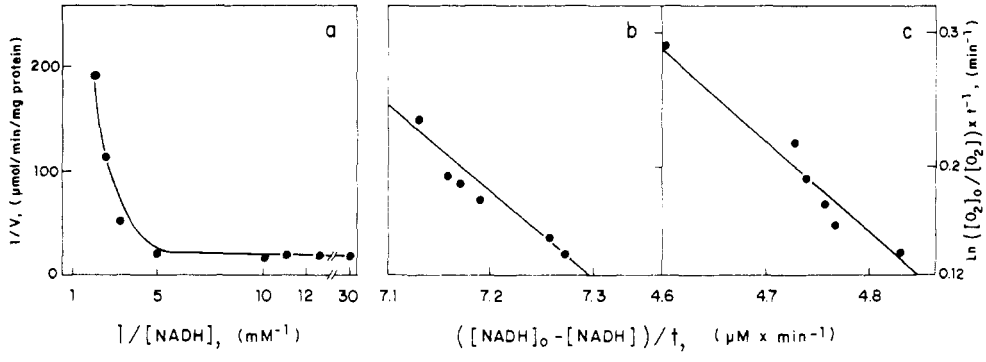

FIGURE 3: Effect of the NADH concentration on the NADH oxidase activity in the absence (b) and in the presence (a and c) of 20 mM KCl. (a) Lineaweaver–Burk plot of the initial rate of NADH oxidation by brush border membranes. Experimental details were the same as those in Figure 1 caption, except that variable amounts of NADH were added. (b and c) The data were obtained as described under Methods for the estimation of the kinetic parameters. The least-squares method was used to draw the lines. The correlation coefficiens were 0.965 (a) and 0.923 (b). The computed kinetic parameters were as follows: K_m = 1.42 (b) and 1.87 μM (c); V_{max} = 0.064 (b) and 0.096 μmol of NADH/(min mg of protein) (c). For experimental details, see Figure 1 caption.

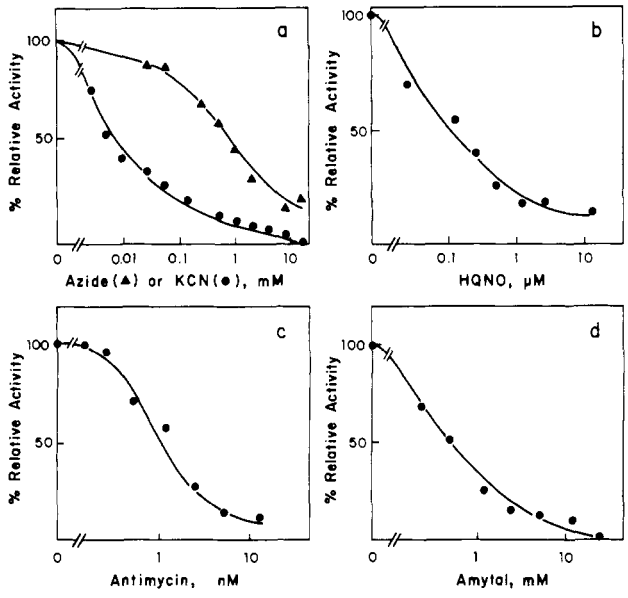

FIGURE 4: Effect of several respiratory inhibitors on the brush border NADH oxidase activity. For experimental details, see Figure 1 caption, except that the reaction was started by addition of NADH up to a concentration of 100 μM and the reaction mixture was incubated for 10 min at 30 °C before the addition of NADH.

Furthermore, it seems to be due only to the reduced form of the nucleotide.

At low NADH concentrations the initial reaction rate could not be reliably estimated because the amount of added substrate changed rapidly. Therefore, the dependence of the activity of NADH concentrations below 50 μM was drawn from the spectrophotometer recorder trace of NADH concentration vs. time which fitted the integrated form of the Michaelis–Menten equation. The apparent K_m for NADH, 1.5–2 μM, was not affected by KCl which caused only an increase of the V_{max} (parts b and c of Figure 3).

Inhibitors of the Brush Border Membrane NADH Oxidase Activity. The effect of some electron transport inhibitors described for other NADH oxidase systems was tested on the brush border NADH oxidase activity. The patterns of inhibition by KCN, HQNO, antimycin, amytal, and sodium azide (Figure 4) were similar to those described for mitochondria (Wainio, 1970). On the contrary, atebrin differently affected brush border and mitochondrial preparations. This compound, described as an inhibitor of the NADH oxidase activity from plasma membrane (Low & Crane, 1978), inhibits almost 100% the NADH oxidation catalyzed by brush border membranes and only 35% the corresponding mitochondrial activity. Besides, atebrin concentrations which did not affect such an activity in mitochondria impaired about 60% the activity of the brush border vesicles (Figure 5). Rotenone was also tested, and at concentrations as low as 50 nM the rate of NADH oxidation remained yet undetectable. No inhibition by HQNO, antimycin, and rotenone was detected when the membranes were stored at −30 °C and frozen simply by putting them in the refrigerator.

Cytochrome System of the Brush Border Membrane. In a previous paper (García et al., 1978) the dithionite reduced minus oxidized optical absortion spectrum of a brush border membrane suspension has been reported. The location of the maxima between 400 and 650 nm (Figure 6, trace b) suggested the presence in these membranes of cytochromes of types *a*, *b*, and *c*. The difference spectrum of membrane suspension, prepared according to the modified procedure reported in this paper, showed similarly located maxima (Figure 6, trace a).

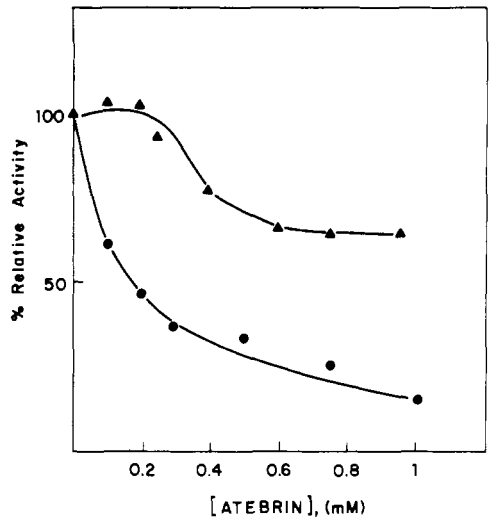

FIGURE 5: Inhibition by atebrine of the NADH-dependent oxygen comsuption. The reaction was started by addition of 300 μmol of NADH to 2 mL of 20 mM KCl, 260 mM mannitol, 3 mM Hepes–Tris (pH 7.4). (●) 0.4 mg of protein/mL of brush border membranes; (▲) 0.4 mg of protein/mL of kidney mitochondria.

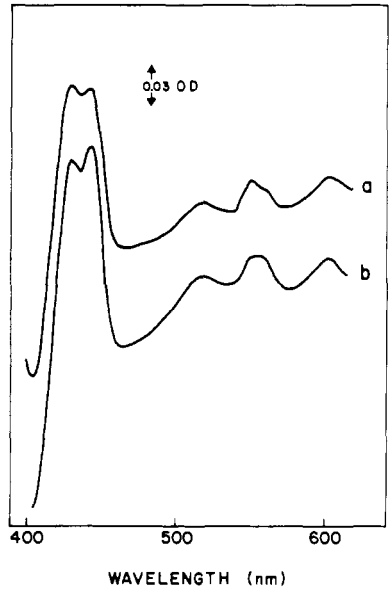

FIGURE 6: Dithionite reduced minus oxidized difference spectra. (a) Brush border membranes prepared as indicated under Methods and (b) as indicated in García et al. (1978). The membranes were suspended for the measurement in 300 mM mannitol and 3 mM Hepes–Tris (pH 7.4) at a concentration of 7.1 mg of protein/mL.

However, the maximum at 550 nm, which can be attributed to the band of *c*-type cytochromes, appeared clearly increased and this independent of the freezing procedure employed for the storage of the membranes. It has been reported (Berezney & Crane, 1972) that cytochrome *c* is loosely attached to the membrane, from which it can be extracted by increasing the ionic strengh of the suspending medium. According to that, the treatment of the brush border membranes for 2 h with 150 mM KCl originated a decrease of the 550-nm maximum in the reduced minus oxidized spectrum, which became then identical with that of membranes prepared by the previous method (Figure 6b; García et al., 1978). The difference spectrum of the supernatant clearly shows this of a single cytochrome type, with maxima at 416, 516 and 547 nm, so identifiable as a *c*-type cytochrome. It seems, therefore, that the modification of the spectrum of the brush border vesicles following the changes in the preparative procedure can be

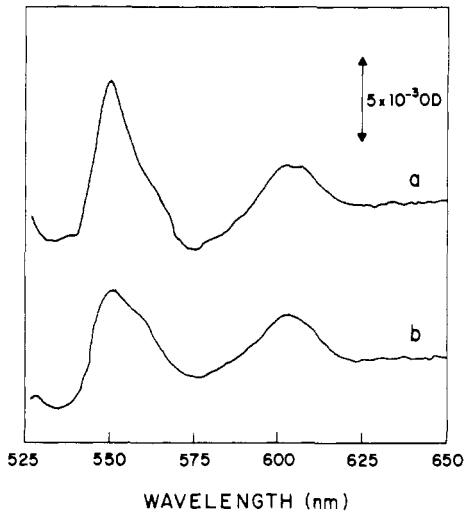

FIGURE 7: Dithionite reduced minus oxidized difference spectrum of a mitochondrial (a) and a brush border (b) membrane suspension. The protein concentration was in both cases 1 mg/mL. Experimental details were as in Figure 6.

Table I: Cytochrome Content of the Renal Brush Border and Kidney Mitochondria[a]

cyto-chrome	renal brush border		kidney mitochondria	
	nmol/mg	av ratio	nmol/mg	av ratio
aa_3	0.233	4	0.199	4
b	0.109	1.87	0.122	2.45
c_1	0.172	2.95	0.152	3.06
c	0.217	3.73	0.320	6.43

[a] Dithionite reduced minus oxidized difference spectra were carried out in 2 mL of 300 mM mannitol and 3 mM Hepes-Tris (pH 7.4) at a membrane protein concentration of 1 mg/mL. No difference in the cytochrome content was found when ferricyanide was added to the oxidized cuvette. For the average ratio calculation, the cytochrome aa_3 content was considered as 4. Calculations were made as described by Vanneste (1966).

attributed to the removal of a *c*-type cytochrome from the membranes, due to the effect of the KCl in the preparation buffer.

In order to quantify the approximate level of each cytochrome type in the brush border membranes, we have followed the method developed by Vanneste (1966) for mitochondrial cytochromes. The similar location of the maxima on the difference spectra of both membrane preparations (Figure 7) seems to allow the use of the wavelength pairs employed in the quoted paper (Vanneste, 1966). In addition, we have assumed that the extinction coefficients for the particular cytochromes are similar in both systems. Keeping in mind these limitations, the calculations reflect clear differences in the level of cytochrome *c* between membrane and mitochondria preparations (Table I). When the same method is applied for the calculation of the cytochrome content of the membrane after the treatment with KCl, the ratio of cytochrome *c* to *a* was 0.25 instead of 0.93 as calculated from Table I for the nontreated membranes.

Effect of NADH and of the Inhibitors of the NADH Oxidase on the Redox State of the Cytochromes. Trace b in Figure 8 indicates that NADH causes an extensive but not complete reduction (cf. trace a) of the brush border vesicle membrane cytochromes under anaerobiosis. The reduction usually reached its maximum level 3 min after the addition of NADH and did not progress beyond during the following 20 min. Quantitative analysis of the spectrum shows that between 30 and 40% of cytochromes *b* and c_1 remained oxidized, and cytochromes aa_3 and *c* were almost fully reduced. The reduction was also completely reversed by oxygen (trace c), suggesting that at least part of the reducing equivalents of NADH are carried to oxygen through the cytochromes.

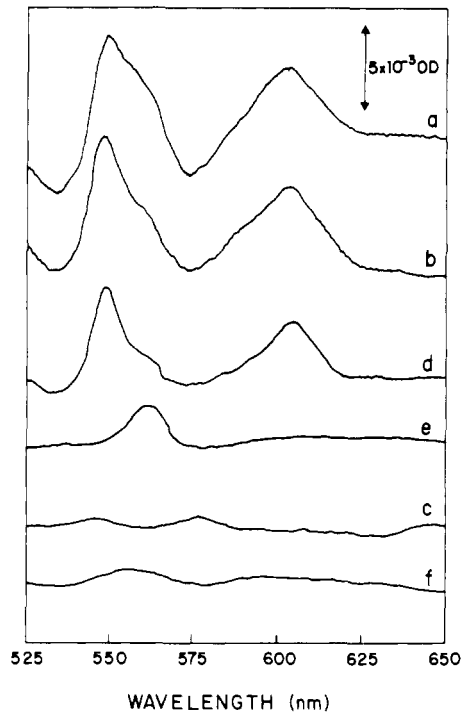

FIGURE 8: Difference spectra of a brush border membrane suspension (1.5 mg/mL) under several conditions. (a) Dithionite reduced. (b–f) Spectra in the presence of NADH (100 μM) under the following conditions: (b) without oxygen; (c) same as (b) after oxygen addition; (d) 10 mM KCN; (e) 5 μM antimycin or 12.5 μM HQNO; (f) 100 nM rotenone or 12.5 mM amytal. Experimental conditions were as in Figure 6.

In the presence of oxygen and KCN at concentrations which impaired oxygen reduction (Figure 8, trace d), NADH also induced a nearly complete reduction of cytochromes aa_3 and *c* (about 80%). However, only approximately 40% of cytochrome *b* appeared to be reduced and cytochrome c_1 was not detectably reduced. According to these data, it seems reasonable to admit that cyanide impaired oxygen reduction by hindering the transport of electrons from cytochromes aa_3 and *c* and partially from cytochrome *b* to this terminal acceptor.

Quantitative analysis of the spectrum obtained when the vesicles are incubated in the presence of oxygen, NADH, and antimycin or HQNO (Figure 8, trace e) shows that only cytochrome *b* is reduced, and this is so to 50% of its total level. According to these data, HQNO and antimycin would impair the transfer of electrons from cytochromes of *b* type to the acceptors.

It seems reasonable to admit according to Figure 8 (trace f) that rotenone and amytal impaired the brush border NADH oxidase system by hindering the electron transport between the NADH dehydrogenase and the pool of cytochromes.

Discussion

The results reported in the present paper lead to the proposal that the brush border cytochromes are constituents of an electron transport system which connects a NADH dehydrogenase with an oxygen reductase, the functionality of such a system being strongly dependent on its cytochrome *c* content.

The incomplete reduction of cytochromes *b* and c_1 by NADH under anaerobiosis and in the presence of the reported inhibitors may be explained by assuming separate pools of

cytochromes with different accessibilities to oxygen and to NADH. Alternatively, it is also possible to propose that all the cytochromes are involved in a single system of electron transport. It should be noted that similar phenomena of incomplete reduction of cytochromes have been observed in the respiratory chain of mitochrondria (Slater, 1958, 1973; Rieske, 1976).

The cytochrome systems of brush border and mitochondria preparations show strong similarities. However, the brush border membrane suspension also presents clear differences with a mitochondrial one. We have reported that in certain conditions freeze-thawing renders NADH oxidase activity of the brush border preparations insensitive to inhibition by antimycin, rotenone, and HQNO (see above). This effect would not be expected of mitochondria. It has also been described (Benavides et al., 1980) in the presence of a β-hydroxybutyrate dehydrogenase activity in the brush border membranes. Although this is also present in the mitochondrial inner membrane, its kinetic constants, thermolability, and substrate accessibility are different in both preparations (Benavides et al., 1980). The inhibitory effect of atebrin on the NADH oxidase system also proves that functionally both brush border and mitochondria preparations are different. Finally, the results of the analysis of marker enzymes must be pointed out (see above). All together, these differences seem to authorize the exclusion of an appreciable mitochondrial or microsomal contamination to account for the presence of cytochromes in the brush border vesicle preparation.

When we reported the existence of cytochromes in the brush border vesicles (García et al., 1978), we advanced the proposal that electron transfer along such a cytochrome system could be more or less directly involved in the energization of the active transport systems required for the physiological function of the kidney. The data presented in this paper, together with the demonstration of transport systems coupled to NADH oxidation in the brush border plasma membrane preparations (García et al., 1980), are consistent with a previous hypothesis (García-Sancho et al., 1977). However, this electron transport system does not seem to be coupled with ATP synthesis since the brush border membranes do not have oligomycin-sensitive ATPase activity.

Acknowledgments

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