Coupling between Reduced Nicotinamide Adenine Dinucleotide Oxidation and Metabolite Transport in Renal Brush Border Membrane Vesicles†

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ABSTRACT: In a previous communication [Giménez-Gallego, G., Benavides, J., García, M. L., & Valdivieso, F. (1980) *Biochemistry* (preceding paper in this issue)] we have reported the ocurrence of a NADH oxidase activity in the renal brush border membranes. The brush border membranes can utilize the energy from the oxidation of NADH to drive the transport of amino acids (aspartic and glutamic acids), organic acids, and the lipophilic cation tetraphenylphosphonium (TPP). The coupling between NADH oxidation seems to be due to the formation of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) as indicated by the effect of specific ionophores. This system may be implicated in the reabsorption process in the renal tubules and in the maintenance of the $\Delta\bar{\mu}_{H^+}$ (positive and acidic in the luminal side) previously described in the renal tubules "in vivo".

Recently, it has been shown that NADH dehydrogenase activities not due to microsomal or mitochondrial contamination are present in mammalian cell plasma membrane [for a review, see Low & Crane (1978) and also Kilberg & Christensen (1979)]. However, electron transfer from NADH to oxygen was not demonstrated the electron acceptor being cytochrome *c* or an artificial compound.

In a previous communication we described a cytochrome system in renal brush border membranes which could be partially reduced by NADH (García et al., 1978). When the membranes are prepared and suspended in a low ionic strength medium instead of the KCl one, NADH is able, under appropriate conditions, to reduce the cytochrome chain and NADH dehydrogenase activity is inhibited by rotenone and other metabolic inhibitors (Giménez-Gallego et al., 1980).

Although there is some indication of an active amino acid uptake dependent on redox reactions in plasma membrane, there is not conclusive evidence for the energization of metabolic transport by NADH oxidation (García-Sancho et al., 1977).

In bacterial plasma membrane, however, Ramos & Kabak (1977) have described systems able to use energy from the oxidation of metabolic substrates to buildup a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] which would drive the accumulation of several metabolites.

Evidence is presented in this report for the energization of metabolic transport by NADH oxidation in renal brush border membrane vesicles. This energization appears to be due to the formation of an electrochemical proton gradient. These results are discussed here with respect to their physiological signficance in the renal function.

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 0.5 M sucrose prepared with double-distilled water, which was previously filtered through Millipore filters (0.22 μm) in order to prevent possible bacterial contamination.

Membranes prepared in 0.5 M sucrose were suspended in a medium containing 300 mM mannitol and 20 mM Hepes–Tris (pH 7.4) and centrifuged at 27000*g* for 5 min, and 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 same medium, frozen in liquid N_2, and stored at −80 °C.

Protein Determination. Protein was determined according to the method of Lowry et al. (1951) using crystalline bovine serum albumin as standard.

Transport Measurement. Brush border membrane vesicles stored at −80 °C were quickly thawed and preincubated at 25 °C for 2 min at a final concentration of 1 mg/mL. Afterwards the radioactive substrate was added. At different times aliquots of 100 μL were diluted in 5 mL of ice-cold 0.8 M NaCl and immediately filtered through Millipore filters (1.2-μm pore size). The filters were washed twice with 5 mL of ice-cold solution. The dilution, filtration, and washing procedures were carried out within 15 s.

The filters were dried at 60 °C and placed in microvials, and their radioactivity content was measured by liquid scintillation after addition of 2 mL of the following medium: 5.5 g of 2,5-diphenyloxazole and 68.6 mg of 1,4-bis[2-(4-methyl-(5-phenyloxazolyl)]benzene in 1000 mL of toluene–Triton X-100 (2:1 v/v).

Materials

[^{3}H]Tetraphenylphosphonium bromide was a generous gift from Dr. S. Ramos (Roche Institute, Nutley, NJ). All other radioactive substrates were purchased from the Radiochemical Centre, Amersham. Valinomycin, CCCP, rotenone, and NADH were provided by Sigma Chemical Co., St. Louis, MO. Nigericin was kindly provided by Lilly Laboratories.

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[1] Abbreviations used: $\Delta\psi$, membrane potential; ΔpH, pH gradient; $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TPP, tetraphenylphosphonium.

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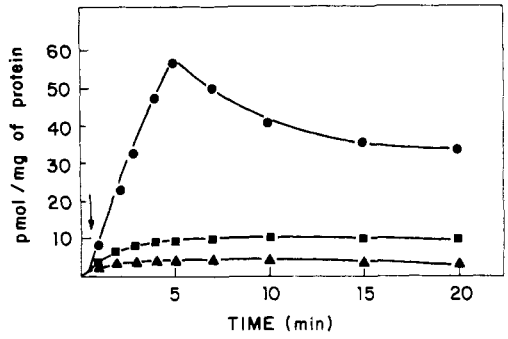

FIGURE 1: L-Aspartate uptake by renal brush border membrane vesicles. The membranes were preincubated for 2 min in a medium containing 260 mM mannitol, 20 mM KCl, and 3 mM citrate–Tris at a final pH of 6. At time zero 6.5 μM L-[U-^{14}C]aspartate with a specific activity of 216 mCi/mmol was added. (■) Control; (●) 360 nmol of NADH; (▲) 10 nmol/mL rotenone in the presence or absence of 360 nmol/mL NADH. The arrow indicates when the NADH was added.

Results

The brush border membrane fraction was of high purity as indicated by previously reported analyses of marker enzyme activities (García et al., 1978; Benavides et al., 1980; Giménez-Gallego et al., 1980).

[U-^{14}C]Aspartate Uptake Is Dependent on NADH Oxidation. When NADH is added to the membrane suspension, there is a stimulation of the aspartate uptake so that at 6 min the accumulation is 7 times larger than that of the control without NADH (Figure 1). After this time an efflux of aspartate is observed.

The NADH effect on the aspartate uptake can be avoided by rotenone (Figure 1) which is also an inhibitor of brush border membrane NADH oxidase activity (Giménez-Gallego et al., 1980). This inhibition agrees with Johnstone's observation that rotenone depresses amino acid transport in Erlich cell plasma membrane (Johnstone, 1976). Rotenone reduces aspartate uptake to levels even lower than that of the control without NADH. This fact may be explained by the existence of an unknown endogenous reductant in the brush border preparation.

Effect of Metabolic Inhibitor. The NADH-dependent aspartate uptake is almost totally abolished by the addition of CCCP, a proton ionophore, suggesting that the energization of the membrane for active transport is due to the formation of a H^+ electrochemical gradient (Figure 2). Other ionophores, such as nigericin, which collapses ΔpH in the presence of K^+ through a K^+/H^+ antiport, or valinomycin, a K^+ mobile carrier, which collapses $\Delta\psi$, also prevent the accumulation of aspartate but to a lower extent than that of CCCP.

Effect of pH and Na^+ and K^+ Concentration. All these experiments have been carried out at pH 6 since at this pH the activation of the uptake by NADH is maximum, this effect being completely eliminated at any pH higher than 7 or lower than 5.

The presence of K^+ in the medium stimulates the rate of aspartate accumulation as shown in Figure 3. This stimulation is highest at 20 mM KCl and may be due to the effect of KCl on the NADH oxidation (Giménez-Gallego et al., 1980), which is also maximum at this concentration. It should be noted that in the presence of rotenone no effect of Na^+ or K^+ was found.

Uptake of Other Compounds. The accumulation of other compounds has also been tested. Only acidic metabolites such as β-hydroxybutyrate and glutamate (Figure 4) or lactate (Figure 5a) are accumulated in a NADH oxidation-dependent fashion. Neutral amino acids such as glycine, alanine, or

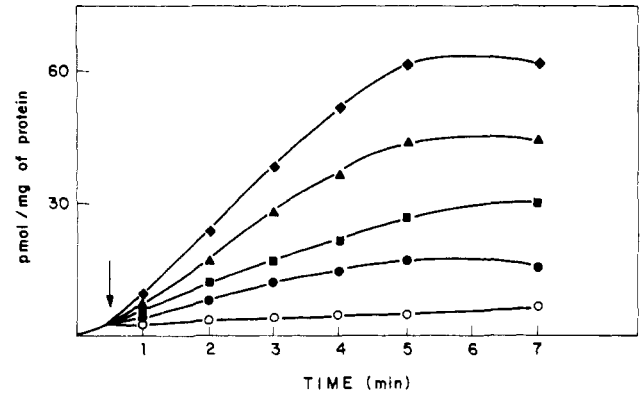

FIGURE 2: Effect of metabolic inhibitors on L-aspartate uptake by renal brush border membrane vesicles. The membranes were preincubated for 2 min in the presence of the inhibitor before addition of the L-[U-^{14}C]aspartate at time zero. (♦) Control; (▲) valinomycin, 4 μM; (■) nigericin, 0.4 μM; (●) CCCP, 20 μM; (○) rotenone, 10 μM. Other experimental conditions were as in Figure 1.

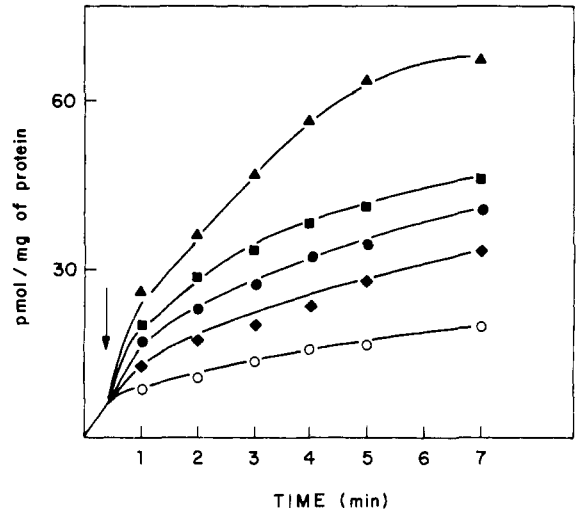

FIGURE 3: Effect of KCl and NaCl on L-aspartate uptake by renal brush border membrane vesicles. The experiments were carried out at several KCl concentrations. The medium osmolarity was kept constant at 300 mM with mannitol. (●) No KCl; (▲) 20 mM KCl; (■) 40 mM KCl; (♦) 30 mM KCl; (○) 10 μM rotenone at 0, 20, 40, and 80 mM KCl. Other experimental conditions were as in Figure 2.

phenylalanine and basic amino acids such as arginine are not taken up under the same conditions. However, lipophilic cations such as TPP can be accumulated by the oxidation of NADH (Figure 5b). This fact may be interpreted as the creation of a transmembrane potential (negative inside) that would drive the uptake of TPP. In the same way the accumulation of weak acids such as lactate or β-hydroxybutyrate may be interpreted as the creation of a pH gradient (alkaline inside). The very small accumulation of these compounds when both NADH and rotenone were absent demonstrates that NADH actually stimulates the uptake.

Discussion

The stimulation of aspartate uptake by NADH seems to be due to the function of the NADH oxidation system existing in the brush border membranes (Giménez-Gallego et al., 1980). This system appears to be involved in the energization of metabolic transport through the formation of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$), as demonstrated by the experiments with uncouplers and ionophores.

The effect of Na^+ and K^+ on the aspartate uptake may be explained by an effect on the NADH oxidation (Giménez-

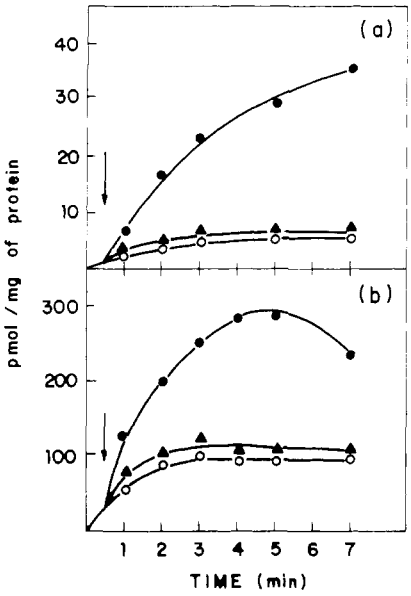

FIGURE 4: L-Glutamate (a) and D(−)-β-hydroxybutyrate (b) uptake by renal brush border membrane vesicles. After 2 min of preincubation, the radioactive substrate was added at time zero. The concentration of L-[U-^{14}C]glutamate was 3.45 μM (285 mCi/mmol) and that of D(−)-β-hydroxybutyrate was 45 μM (22 mCi/mmol). The incubation medium was 300 mM mannitol and 3 mM citrate–Tris at a final pH of 6. (▲) Control; (●) 360 nmol/mL NADH; (○) 10 nmol/mL rotenone in the presence or absence of 360 nmol/mL NADH.

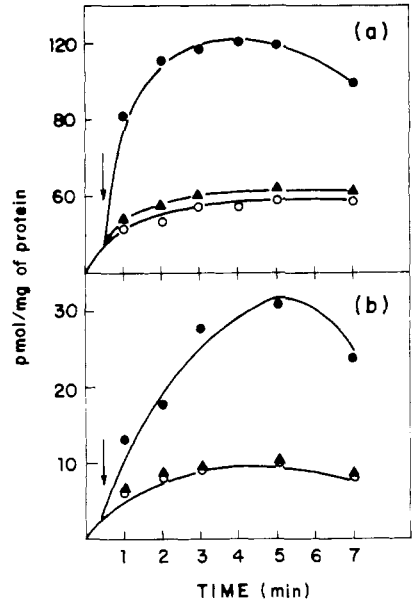

FIGURE 5: L-Lactate (a) and tetraphenylphosphonium (b) uptake by renal brush border membrane vesicles. After 2 min of preincubation, the radioactive substrate was added at time zero. The concentration of L-[U-^{14}C]lactate was 21 μM (50 mCi/mmol) and that of [^{3}H]TPP was 7 μM (200 mCi/mmol). The incubation medium was 300 mM mannitol and 3 mM citrate–Tris at a final pH of 6. (▲) Control; (●) 360 nmol/mL NADH; (○) 10 nmol/mL rotenone in the presence or absence of 360 nmol/mL NADH.

Gallego et al., 1980), even though direct action on the carrier cannot be disregarded. However, the system described here is different from the Na^+-dependent aspartate transport as demonstrated by the stimulation of the uptake by NADH in the complete absence of Na^+. Moreover, the accumulation of weak acids such as lactate and β-hydroxybutyrate and a lipophilic cation such as TPP is a clear indication that the oxidation of NADH originates an electrochemical proton gradient across the brush border membrane. Since there is good evidence for the physiological pH and potential gradient across the brush border membranes (alkaline and negative inside) (Sacktor, 1977), we suggest the possibility that the operation of the electron transport chain in brush border membranes could be responsible for this electrochemical proton gradient, although other mechanisms proposed previously such as the HCO_3^--ATPase (Sacktor, 1977) could also be involved. This proton gradient could be utilized under physiological conditions to build up a Na^+ gradient through the Na^+/H^+ antiport previously described in brush border membranes (Murer et al., 1976). This antiport associated with the proton gradient could be an alternative mechanism to maintain the Na^+ gradient necessary for Na^+-dependent transport processes. The advantage of this mechanism over the Na^+, K^+-ATPase should be its ability to create a Na^+ gradient just where it is going to be utilized, the luminal membrane; Na^+, K^+-ATPase is mainly localized in the laterobasal membrane of the renal tubules (Sacktor, 1977). A mechanism for proton secretion by gastric mucose driven also by electron transport has been propossed by Hersey (1974, 1977).

The observation that neither the Na^+ gradient nor the energy from ATP can fully explain in some cases the energization of metabolic transport across plasma membranes (Christensen et al., 1973; Schafer et al., 1977; Banay-Schwartz et al., 1976) has stimulated the search for other energy sources for metabolic transport. Thus, good evidence of a plasma membrane amino acid transport system dependent on redox reactions has been obtained in Erlich cells (García-Sancho et al., 1977). Other attempts to demonstrate metabolite transport coupled to NADH oxidation in isolated plasma membrane have been reviewed by Crane (Crane et al., 1979).

The system described here represents a new means of energizing transport in mammalian plasma membrane, dependent on redox reactions instead of ATP hydrolysis or a Na^+ gradient and similar to those described in bacteria plasma membrane (Ramos & Kaback, 1977).

Acknowledgments

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Kinetic Mechanism of Horse Liver Alcohol Dehydrogenase SS†

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ABSTRACT: The kinetic mechanism of SS isozyme of horse liver alcohol dehydrogenase is shown by initial velocity and product inhibition studies to be asymmetrical, being random for ethanol oxidation and compulsory ordered for acetaldehyde reduction. Enzyme isomerization seems to account for the asymmetry in the mechanism. In its interaction with NADH, the SS isozyme resembles classical alcohol dehydrogenase; consequently, the maximal velocity in the direction from ethanol to acetaldehyde appears to be determined by the rate of NADH dissociation. In the direction from acetaldehyde to ethanol, the enzyme isomerization step appears to limit the maximal velocity.

The kinetic mechanism of classical horse liver alcohol dehydrogenase [a preparation containing 60–80% of EE isozyme[1] and henceforth referred to as (EE) isozyme] was determined by several investigators (Theorell & Chance, 1951; Theorell & McKinley-McKee, 1961; Dalziel, 1957; Dalziel & Dickinson, 1966a,b) and that of homgeneous EE isozyme was determined by Hanes et al. (1972). The kinetic mechanism of (EE) isozyme is best described as a special case of a symmetrical ordered bi-bi mechanism in which the dissociation of the product–coenzyme, rather than the interconversion of the productive ternary complexes, is rate limiting (Cleland, 1970; Dalziel, 1975). The formation of the productive ternary complexes has been verified experimentally by Wratten & Cleland (1963, 1965), Theorell & Yonetani (1962), and Theorell & Tatemoto (1970), the latter employing homogeneous EE isozyme. The mechanism deviates from the compulsory ordered mechanism when initial velocity studies are performed with secondary alcohols (Dalziel & Dickinson, 1966b). Evidence for alcohol oxidation via a partially random mechanism was found in isotope exchange studies (Silverstein & Boyer, 1964) and in initial velocity studies using a wide range of substrate concentrations (Hanes et al., 1972). Hence, the kinetic mechanism of EE isozyme can be more precisely described as partially random for alcohol oxidation and compulsory ordered for aldehyde reduction with the rate of product–coenzyme dissociation as the rate-limiting step.[2]

The EE and SS isozymes are well suited for structure–function studies in view of the small amino acid sequence difference between the E and S subunits (Jörnvall, 1970a,b). Both subunits when present as a part of a dimeric enzyme molecule possess the wide substrate specificity which is characteristic of alcohol dehydrogenase, but only the S subunit can catalyze reversible redox reactions between 3-ketosteroids and 3β-hydroxysteroids (Pietruszko et al., 1966; Theorell et al., 1966). The steroid activity appears to be the result of a single amino acid difference between the E and S subunits (Eklund et al., 1976). The kinetic mechanism of the acetaldehyde–ethanol interconversion catalyzed by SS isozyme has not been determined.[3] Elucidation of this functional aspect of SS isozyme is necessary for comparison with EE isozyme and for determining structure–function relationships between these isozymes.

Experimental Procedures

NADH (grade III) was purchased from Sigma Chemical Co., St. Louis, MO. NAD (grade II) obtained from Boeh-

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[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; (EE) isozymes, classical preparations of horse liver alcohol dehydrogenase; EE and SS isozymes, single, isozyme preparations; ϕ_O, ϕ_A, ϕ_B, and ϕ_{AB}, kinetic coefficients referring to the reaction in the direction from ethanol to acetaldehyde; ϕ'_O, ϕ_Q, ϕ_P, and ϕ_{PQ}, kinetic coefficients referring to aldehyde reduction. Cleland nomenclature is employed throughout: K_a, K_m(NAD); K_{ia}, dissociation constant for NAD from the enzyme·NAD binary complex; K_b, K_m(ethanol); K_{ib}, dissociation constant for ethanol from the enzyme·ethanol binary complex; V_1, turnover number × (active site)$^{-1}$ × s^{-1} at saturating NAD and ethanol. K_q, K_m(NADH); K_{iq}, dissociation constant for NADH from the enzyme·NADH binary complex; K_p, K_m(acetaldehyde); V_2, turnover number × (active site)$^{-1}$ × s^{-1} at saturating NADH and acetaldehyde. V_1/(Et) and V_2/(Et) refer to saturating ethanol and acetaldehyde at 500 μM NAD and 170 μM NADH.

[2] This work is a part of a Ph.D. thesis of C. N. Ryzewski.

[3] The catalytic mechanism of an isozyme of alcohol dehydrogenase described as SS isozyme has been published by Dworschack & Plapp (1977). The properties and mechanism of their enzyme are so different from ours that to save space we have decided not to compare our data with theirs. We also possess evidence (C. N. Ryzewski and R. Pietruszko, unpublished experiments) proving that their and our data refer to different isozymes.

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