Biochimica et Biophysica Acta 816 (1985) 68–76
Elsevier

BBA 72599

Protein-mediated chloride-phosphate and lactate-lactate exchange in cytoskeleton-free vesicles budded from rabbit erythrocytes

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(Received December 17th, 1984)

Key words: Lactate transport; Transport protein; Erythrocyte membrane

Spectrin-free budded vesicles from rabbit erythrocytes (Leonards, K.S. and Ohki, S. (1983) Biochim. Biophys. Acta 728, 383–393) exchange intravesicular L-[^{14}C]lactate for extravesicular L-lactate and intravesicular [^{36}Cl]chloride for extravesicular phosphate with inhibitor sensitivity consistent with what is seen in intact cells. The time-course of these fluxes is faster than for intact cells, but is somewhat slower than predicted from surface to volume ratios. Labelling with tritiated 4,4′-diisothiocyanyl-2,2′-dihydrostilbenedisulfonate (H_2DIDS) at concentrations which selectively inhibit inorganic anion exchange or specific lactate exchange supports the involvement of a 93–110 kDa (band 3) polypeptide in anion transport and a 40–50 kDa polypeptide in lactate transport across these vesicle membranes. Since the budded vesicles have a markedly simplified protein profile on electrophoresis, their isolated membranes represent a preliminary stage in the purification of these transport proteins in which structure and function appear to be preserved.

Introduction

A specific lactate transport system appears to be a common feature of the erythrocytes in rabbits, humans, and other mammals [1–4] as well as in Ehrlich ascites tumor cells [5,6] and thymocytes [7,8]. The kinetics and inhibitor sensitivity of this transport system are well characterized for red blood cells [1]. Rabbit erythrocytes have a high capacity for lactate transport [2], suggesting that this transport protein is abundant in these cells [9]. The intact erythrocyte membrane is a heterogeneous combination of lipids and intrinsic and extrinsic proteins. This makes the problem of identifying a particular polypeptide responsible for mediating specific lactate exchange a formidable one. Recent evidence from our laboratory using chemical labelling with [^{3}H]H_2DIDS in concentrations that inhibit specific lactate exchange supports the association between a 40–50 kDa polypeptide of the rabbit erythrocyte membrane and lactate transport [9,10].

Protein-mediated inorganic anion exchange is also a common feature of erythrocytes. This system plays an important role physiologically in the chloride-bicarbonate exchanges that occur as red blood cells circulate beteen lungs and tissues. A large body of kinetic and chemical labelling methods has long supported the role of band 3 [11], a 93–110 kDa glycoprotein, in anion transport [12–14] in human erythrocytes. Band 3 from human erythrocyte membranes has been isolated in detergent-solubilized form and reconstituted into liposomes, which are then able to catalyze anion

Abbreviations: H_2DIDS, diisothiocyanyldihydrostilbenedisulfonate; iBCLA, isobutylcarbonyllactyl anhydride; pCMBS, *p*-chloromercuribenzenesulfonate; PMSF, phenylmethylsulfonyl fluoride; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

exchange [15–18]. Band 3 is accepted as the anion carrier in human red blood cells, and, by inference, in other red blood cells. While the reconstitution of rabbit band 3 has not been reported, other characteristics of a 93–110 kDa rabbit red cell membrane polypeptide strongly suggest that it is also an anion transporter. For example, low concentrations of H_2DIDS strongly inhibit chloride-phosphate exchange; the same concentrations of [^{3}H]H_2DIDS preferentially label rabbit band 3 [9]. Also, data from this laboratory show that fragments of rabbit band 3 of characteristic molecular weight are produced when red cells and red cell membranes are treated with chymotrypsin and trypsin (not shown).

Leonards and Ohki [19–21] recently developed a simple method for obtaining stable, cytoskeleton-free budded vesicles from rabbit erythrocytes. Such vesicles have a simplified membrane protein profile and normal membrane orientation. They potentially offer a significantly simplified system in which to study protein-mediated membrane transport. Rabbit erythrocyte membrane vesicles can be viewed as a rudimentary purification of the intrinsic membrane protein constituents of the parent cells. The vesicle membranes are completely lacking in cytostructural proteins [20], which can comprise almost half of the membrane-associated protein of mammalian erythrocytes [22].

The purpose of this paper is to present experiments which demonstrate the presence and certain characteristics of a specific lactate transporter and an inorganic anion transporter in rabbit erythrocyte membrane vesicles. Despite incubation at 45°C and estrangement from the underlying cytoarchitecture, these transport systems show sensitivity to the same inhibitors as intact cells. The time-course of the lactate-lactate and chloride-phosphate fluxes is faster than in intact cells, but somewhat slower than predicted from surface area to volume ratios. [^{3}H]H_2DIDS-labelling experiments analogous to those done previously on intact cells [9,10], support an association between a 40–50 kDa polypeptide and lactate transport, and between a band 3 polypeptide and chloride-phosphate exchange across membrane vesicles. This paper is the first report of protein-mediated transport for this membrane vesicle system.

Materials and Methods

Fresh rabbit blood was obtained by ear vein phlebotomy from New Zealand white rabbits (EDTA 5 mM anticoagulant). The blood was used within 2 h, and never cooled below room temperature. L-[^{14}C]Lactic acid (90 mCi/mmol) and [^{36}Cl]HCl (0.45 mCi/mmol) were purchased from ICN Radiochemicals. H_2DIDS and [^{3}H]H_2DIDS were prepared in our laboratory [23] according to the methods adapted from Lepke et al. [24] and Levinson et al. [25]. Isobutylcarbonyllactyl anhydride (iBCLA) was prepared in our laboratory [10] according to the method of Johnson et al. [5]. *para*-Chloromercuribenzenesulfonate (pCMBS) was purchased from Sigma.

Vesicle preparation. Budded vesicles from rabbit erythrocytes were prepared according to the method of Leonards and Ohki [19–21] with several minor variations. Erythrocytes were washed (buffy coat removed) by repeated centrifugation at room temperature in 150 mM NaCl/10 mM Tris-HCl (pH 7.4) (NaCl/Tris). The budding procedure was carried out exactly as described by Leonards and Ohki [20], i.e., by titrating suspended cells with EDTA and $CaCl_2$ at 45°C, except that Tris was used instead of Tricine as the buffer. The progress of budding was monitored microscopically. The resulting vesicles were separated from the parent cells and collected in the aqueous and 30% layers of 40/30% (w/w NaCl/Tris plus 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) discontinuous sucrose gradients following 30 min of centrifugation at 4°C at 900 rpm in the IEC clinical centrifuge, number 215 rotor. The vesicles were washed in NaCl/Tris plus PMSF by centrifugation at $15\,000 \times g$ for 90 s at 4°C. Vesicles were resuspended in NaCl/Tris plus PMSF, and layered on 30% (w/w NaCl/Tris plus PMSF) sucrose and centrifuged at 1800 rpm in the IEC clinical centrifuge for 10 min at 4°C. The vesicles sedimented as a red band at the aqueous/30% interface. They were collected, washed, and stored up to 3 days at 4°C.

Flux studies. Carruthers and Melchior [26] describe a method for measuring [^{14}C]glucose efflux from 0.12 μm diameter protein-lipid vesicles. They use the Eppendorf bench centrifuge to form pellets of vesicles from which efflux is stopped by a

suitable inhibitor at appropriate time-points. The radioactivity in the pellets is counted and plotted versus time to depict the efflux. This approach was adapted for studying L-[^{14}C]lactate efflux and [^{36}Cl]chloride-phosphate exchange in membrane vesicles.

L-[^{14}C]Lactate efflux from vesicles. Vesicles (about 50 μl packed volume) were washed in 150 mM KCl/10 mM sodium phosphate (pH 7.4) (KCl/phosphate), pelleted by centrifugation for 90 s in the Eppendorf bench centrifuge, and resuspended in KCl/phosphate plus 5 mM sodium lactate plus 5 μl L[^{14}C]lactate (0.25 mCi/ml), for a final volume of 0.3 ml. The vesicles were incubated for 15 min at room temperature, after which they were pelleted in the Eppendorf centrifuge. Supernatants were aspirated, leaving about a 50% suspension of vesicles in the loading medium. This was necessary because the vesicles were very cohesive; their instantaneous introduction into the flux medium at time = zero required their not being tightly packed. Aliquots of 5 μl of the loaded vesicle suspension were introduced at time = zero into 0.5 ml of flux medium in Eppendorf tubes on ice. The flux medium was KCl/phosphate with 5 mM sodium lactate. At appropriate time-points, as indicated in Fig. 1, 0.5 ml of ice-cold 150 mM NaCl/20 mM sodium phosphate (pH 6.0)/2 mM pCMBS/0.5 mM phloretin was added to stop the efflux, and the tube was spun immediately for 90 s in the Eppendorf centrifuge. All supernatants were removed by aspiration, leaving a tight red pellet in the tip of the tube. The tip of the tube was removed with a razor blade, placed in a 25 ml scintillation vial with 1 ml distilled H_2O, vortexed, the contents suspended in 10 ml scintillation cocktail, and counted for ^{14}C. Time = zero time-points were obtained by introducing 5 μl of the labelled suspension directly into an ice-cold mixture of 0.5 ml of the flux medium and 0.5 ml of the stop solution, followed by centrifugation and subsequent steps as above.

Trans-effect on L-lactate efflux. L-[^{14}C]Lactate efflux was conducted exactly as above, except that the loading medium was 150 mM KCl/20 mM sodium phosphate (pH 7.4)/5 mM sodium lactate/5 μl L[^{14}C]lactate, final volume 0.3 ml. The flux medium was 150 mM KCl/20 mM sodium phosphate (pH 7.4) with or without 5 mM sodium lactate.

[^{36}Cl]Chloride-phosphate exchange. Inorganic anion transport, which is mediated by band 3 in intact erythrocytes [12–14], was assessed in vesicles by measuring the exchange of intravesicular [^{36}Cl]chloride for extravesicular phosphate. Again, the methodology is adapted from Carruthers and Melchior [26]. Vesicles washed in KCl/phosphate were suspended with KCl/phosphate plus [^{36}Cl]chloride in the proportions 30 μl pellet vesicles to 120 μl KCl/phosphate to 4 μl neutralized [^{36}Cl]HCl (activity 50 μCi/ml). Efflux was initiated by suspending 10 μl aliquots of the loaded vesicle suspension into 0.5 ml of 140 mM sodium phosphate (pH 6) at 15°C in Eppendorf tubes. At appropriate time-points (see Fig. 2), 0.5 ml of 100 μM H_2DIDS in 140 mM sodium phosphate (pH 6), 0°C was added to stop the exchange. The tube was spun immediately for 90 s in the Eppendorf centrifuge, and all subequent steps were exactly as described above for the L-lactate efflux, except that ^{36}Cl was counted instead of ^{14}C. Time = zero time-points were obtained by introducing the aliquot of tracer-loaded vesicles directly into 1.0 ml of ice-cold 50 μM H_2DIDS in 140 mM sodium phosphate (pH 6), followed by centrifugation and subsequent steps as above.

H_2DIDS treatment of vesicles. Vesicles washed in KCl/phosphate were incubated at 37°C, 30 μl vesicles per 1.0 ml volume of KCl/phosphate plus 4 μM H_2DIDS for 80 min, or 25 μM H_2DIDS for 60 min. The vesicles were then washed by centrifugation for 90 s in the Eppendorf bench centrifuge with KCl/phosphate plus 0.2% bovine serum albumin, followed by two washes in KCl/phosphate. The vesicles were then used in flux assays or subsequent labelling experiments. H_2DIDS is known to strongly inhibit chloride-phosphate exchange at 4 μM concentration, but requires 20 μM concentration to inhibit lactate exchange by 50% in rabbit red blood cells [9].

iBCLA treatment of vesicles. Vesicles washed in KCl/phosphate were incubated on ice, 30 μl vesicles per 1.0 ml volume KCl/phosphate plus 20 μM iBCLA (introduced as 1 mg/ml ethanolic solution) for 60 min. Treated vesicles were washed three times in KCl/phosphate. iBCLA is a known selective inhibitor of lactate transport in red blood cells [5,10].

pCMBS treatment of vesicles. Vesicles washed in

KCl/phosphate were incubated at room temperature. 30 μl vesicles per 1.0 ml total volume were incubated in KCl/phosphate plus 2 mM pCMBS for 5 min, then washed three times in KCl/phosphate. pCMBS is a known inhibitor of lactate transport, but has no effect on chloride-phosphate exchange in rabbit red blood cells [27].

Labelling of vesicles by [^{3}H]H_2DIDS. Membrane vesicles were labelled with [^{3}H]H_2DIDS in two ways. Vesicles were either treated directly with this radiolabel, or were budded and isolated from intact cells which were already labelled.

Vesicles were treated with 4 and 25 μM [^{3}H]H_2DIDS using exactly the method described for treating vesicles with unlabelled H_2DIDS. Also, vesicles treated with 4 μM unlabelled H_2DIDS were washed and subsequently treated with 25 μM [^{3}H]H_2DIDS, as described above. The washed, labelled vesicles were lysed osmotically [11] by suspending them in at least 50 vol. of ice-cold 5 mM Tris-HCl (pH 7.4) plus 0.2 mM PMSF. The suspended lysed vesicles were stored on ice for 2–3 h. Membranes were centrifuged at 100 000 × *g* for 60 min at 4°C, and the pellets were reserved. Aliquots of the isolated membranes were solubilized in 4% 80 mM dithiothreitol/120 mM Tris-HCl (pH 6.8)/0.01% Bromphenol blue, and heated 3 min at 100°C. The samples were electrophoresed according to the method of Laemmli [28] on 12 cm polyacrylamide gels (6–18% acrylamide, 5% stacking gel). Lanes were excised and cut into 2.5-mm segments which were digested in 0.8 ml 30% H_2O_2 at 50°C overnight. Radioactivity in the segments was measured by liquid scintillation counting. Prior to slicing, gels were stained in Coomassie blue G and the lanes were scanned spectrophotometrically to permit a comparison to be made between the peaks of radioactivity and molecular weights of standards run on the same gel. Due to the large and variable amount of hemoglobin associated with these membranes even after lysis, an absolute determination of membrane protein was not made.

Alternatively, intact red blood cells were incubated in KCl/phosphate plus 25 μM [^{3}H]H_2DIDS for 60 min at 37°C, washed in KCl/phosphate plus bovine serum albumin, then in NaCl/Tris, and finally in NaCl/Tris plus 0.2 mM PMSF, all at 37°C. The budding process was then carried out exactly as described above. The vesicles were collected from the aqueous phase over 33% (w/w NaCl/Tris plus 0.2 mM PMSF) sucrose following 30 min of centrifugation at 900 rpm in the IEC clinical centrifuge. The vesicles were washed, lysed, solubilized and subjected to electrophoresis as described above. Gel lanes were stained, scanned, sliced and counted for tritium. Some of the labelled parent cells were reserved before the budding procedure. The membranes (ghosts) from these cells were isolated, subjected to electrophoresis, and counted for tritium exactly as described previously [9,10].

Results

Lactate transport in vesicles

The time-course of L-lactate efflux from vesicles is shown in Fig. 1. The efflux was inhibited by 2 mM pCMBS, 20 μM iBCLA and 25 μM H_2DIDS, but not by 4 μM H_2DIDS. These concentrations of the same agents are known to inhibit lactate transport in intact rabbit erythrocytes [1,9,10]. The

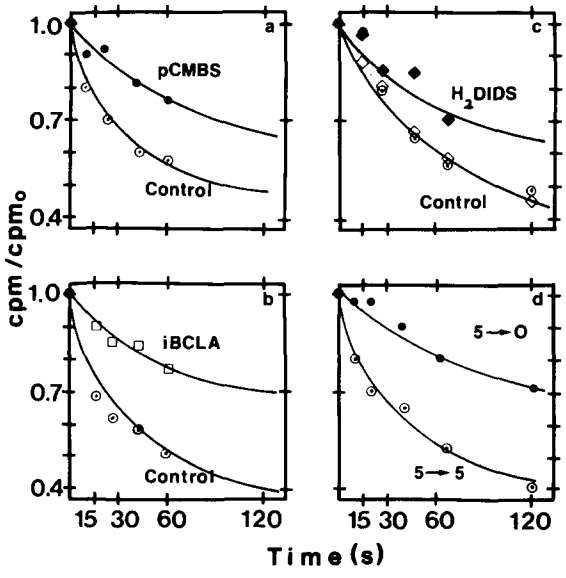

Fig. 1. Efflux of L-[^{14}C]lactate from vesicles. Vesicles treated with various inhibitors are compared with controls (⊙) taken from the same vesicle preparation. (a) 2 mM pCMBS (●), (b) 20 μM iBCLA (□), (c) 4 (◇) and 25 (◆) μM H_2DIDS. The transacceleration is shown in (d), where the upper curve (●) shows the efflux of 5 mM lactate into lactate-free medium, and the lower curve (⊙) shows the efflux of 5 mM lactate into medium containing 5 mM lactate.

efflux of 5 mM lactate into flux medium containing 5 mM lactate was about 4-times faster than the efflux of 5 mM lactate into flux medium without lactate. Under the same flux conditions (0°C, pH 7.4), this transacceleration is also reported for intact rabbit red blood cells [9].

Chloride-phosphate exchange in vesicles

Fig. 2 shows the time-course of the exchange of intravesicular [^{36}Cl]chloride for extravesicular phosphate (15°C, pH 6). This exchange was strongly inhibited by 4 μM H_2DIDS, which left lactate exchange uninhibited (Fig. 1), just as is reported for intact rabbit erythrocytes [9]. Control experiments in which rabbit erythrocytes were incubated at 45°C for 2 h prior to lactate-lactate or chloride-phosphate exchange fluxes showed that these exchange processes were not altered by this heating (data not shown).

Labelling of vesicles by [^{3}H]H_2DIDS

Membranes isolated from vesicles which were treated directly with 4 μM [^{3}H]H_2DIDS and subjected to electrophoresis showed radiolabelling of a 93–110 kDa band with minor labelling of a 40–50 kDa band (Fig. 3). Treatment with 25 μM [^{3}H]H_2DIDS labelled more of the 40–50 kDa band relative to the 93–110 kDa band than the 4 μM [^{3}H]H_2DIDS. When vesicles were treated with 4 μM unlabelled H_2DIDS followed by 25 μM [^{3}H]H_2DIDS, the 40–50 kDa band was again labelled, but the 93–110 kDa band was not. A similar concentration-dependent radiolabelling of

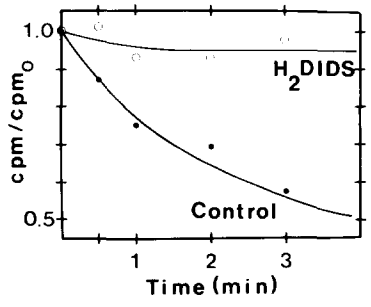

Fig. 2. Exchange of intravesicular [^{36}Cl]chloride for extravesicular phosphate. Vesicles treted with 4 μM H_2DIDS (⊙) are compared with controls (●) taken from the same vesicle preparation. The H_2DIDS strongly inhibits chloride-phosphate exchange in these vesicles.

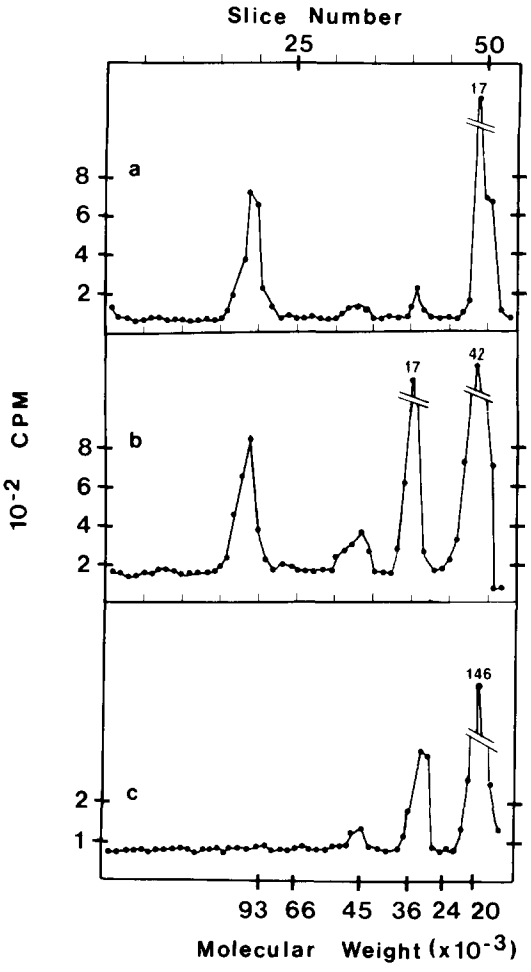

Fig. 3. Direct labelling of vesicles by [^{3}H]H_2DIDS. Vesicles were treated with 4 μM [^{3}H]H_2DIDS (a), 25 μM [^{3}H]H_2DIDS (b), or 4 μM unlabelled H_2DIDS followed by 25 μM [^{3}H]H_2DIDS, as described in Materials and Methods. Vesicle membranes were isolated, solubilized and subjected to electrophoresis. Gel lanes were sliced, solubilized in 30% H_2O_2 and counted for tritium by liquid scintillation counting. Material in each lane is standardized against the area under the absorbance peak at 600 nm for the Coomassie staining of band 3: (a) 1.00, (b) 1.04, (c) 0.41. Molecular weight comparisons are to standards run on the same gel (not shown).

membrane polypeptides is also reported for intact rabbit erythrocytes [9]. Strongly labeled bands at about 17 and 33 kDa are not reported for intact cells, but were observed in vesicles that were directly labelled. These bands were partially dissociable from vesicle membranes by treatment with 1 M NaCl plus 0.1 N NaOH and sonication in a bath sonicator (data not shown), and are probably

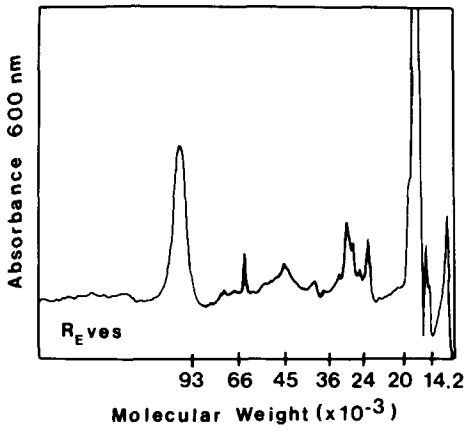

Fig. 4. Coomassie-staining profile of vesicles. The vesicle membranes were isolated, subjected to electrophoresis on the same gel as in Fig. 3, stained, and scanned as described in Materials and Methods.

not membrane-bound [16]. The Coomassie-staining profile of vesicle membranes run on the same polyacrylamide gel is shown in Fig. 4.

When vesicles were made from fresh rabbit erythrocytes pretreated with 25 μM [^{3}H]H_2DIDS, both the 93–110 and the 40–50 kDa bands of the isolated vesicle membranes were labelled (Fig. 5). Ghost membranes isolated from the treated cells prior to budding showed a similar pattern of labelling which has been previously described [9]. The labelling of the 17 and 33 kDa polypeptides was not seen in membranes from vesicles made from prelabelled cells.

Discussion

The inhibitor sensitivity of the specific lactate and the inorganic anion transport shown in Figs. 1 and 2 supports the existence of transport proteins which mediate these processes in the rabbit erythrocyte membrane vesicles. Moreover, the concentration dependence of the inhibition of chloride-phosphate and lactate-lactate exchange by H_2DIDS, and the inhibition of the lactate-lactate exchange by pCMBS and iBCLA, indicates that these transport proteins behave as predicted from inhibition studies done previously on intact cells [9,10]. However, the extent of inhibition of lactate exchange in the vesicles by pCMBS, iBCLA, and H_2DIDS is not as great as previously reported for

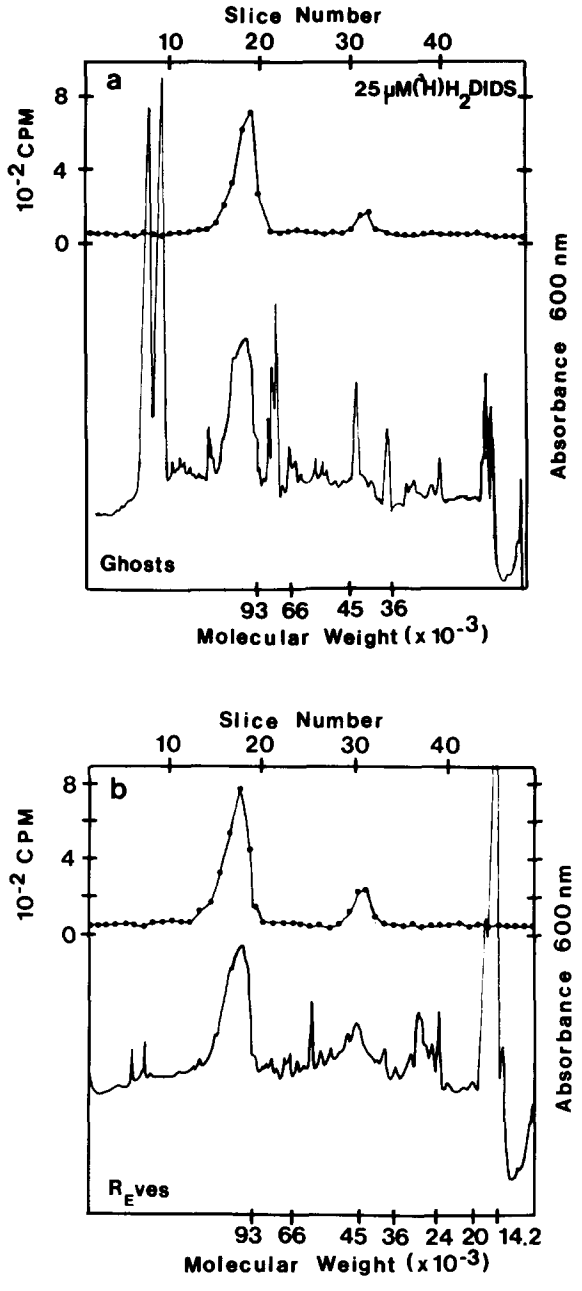

Fig. 5. Labelling of ghosts and vesicle membranes made from rabbit erythrocytes prelabelled with 25 μM [^{3}H]H_2DIDS. Ghosts and vesicle membranes were solubilized and electrophoresed as described in Materials and Methods. Gel lanes were sliced, solubilized in 30% H_2O_2 and counted for tritium by liquid scintillation counting. The radiolabelling patterns of ghosts (a) and isolated vesicle membranes (b), are compared with the Coomassie-staining profile of the respective gel lanes. The pattern of labelling in the ghosts is just as previously reported [10]. This pattern is preserved in the budded vesicles.

intact cells, while the inorganic anion exchange is very strongly inhibited by H_2DIDS in the vesicles. This indicates that some properties of the lactate transporter may be altered in the budding process. The time-course of lactate-lactate and chloride-phosphate exchange is faster in the vesicles than in the intact cells. However, the exchanges are not as fast as predicted from strictly geometric considerations. Fick's first law can be used to show that the half-time of a transport process by diffusion is directly proportional to the ratio of the volume to surface area (V/A) for the enclosing membrane and indirectly proportional to the permeability coefficient (P), a number related to the intrinsic properties of the membrane [29,30]. The V/A for an intact rabbit erythrocyte is about 0.6 μm [31], corrected to 0.4 μm when 32% w/v hemoglobin in the cytoplasm is considered. From electronmicrographs done here (not shown), the size of the washed vesicles used in the flux studies are of estimated diameters 0.2–0.5 μm, consistent with what has been reported by Leonards and Ohki [20]. This gives a V/A of about 0.03–0.09 μm, or an order of magnitude smaller than for intact cells. The half-times of lactate-lactate and chloride-phosphate exchange in intact control cells under the same flux conditions as used for the vesicle controls are characteristically 120 and 270 s, respectively [9,10]. The mean half-times of these fluxes in vesicles are about 90 s (range 70–110 s, $n = 4$) for lactate efflux and 210 s (range 180–240, $n = 4$) for inorganic anion exchange, compared to predicted values, assuming constant P, of about 12 and 27 s. Since the larger vesicles have a greater influence on the overall time-course of the fluxes, these predicted half-time values are probably underestimated. The slowing of the transport might also be accounted for by denaturation of the transport proteins, e.g., by aggregation in the budded membranes. Heating the in situ transport proteins is not alone sufficient to slow the transport, since intact cells which were incubated at 45°C before being used in flux experiments showed no differences in the time-courses of lactate-lactate or chloride-phosphate exchange. However, it is conceivable that when the transport proteins are dissociated from the underlying cytoarchitecture in a hyperfluid (45°C) lipid environment, they could form aggregates with lower transport activities. Another possibility is that the numbers of functional lactate and inorganic anion transporters per unit membrane area might be reduced during the budding process. The range in half-times might be accounted for by the same factors, i.e., by differences in mean vesicle size, denaturation, or number per unit area of functional transport proteins in a given preparation.

The labelling experiments shown in Figs. 3 and 5 indicate that chloride-phosphate and lactate-lactate exchange are probably mediated by polypeptides which are unaltered in their electrophoretic mobilities by the budding process. Membranes from vesicles made from cells which are prelabelled with [^{3}H]H_2DIDS have band 3 and 40–50 kDa polypeptides labelled on electrophoresis. Since the same labelled bands are associated, respectively, with inorganic anion transport [12–14] and specific lactate transport [9,10] in the intact parent cells, and since these transport processes are present and susceptible to the same inhibitors in the vesicles, it is reasonable to assert that these bands are conserved structurally and (to some extent) functionally in the budded vesicles. Isolated vesicles which are labelled directly with [^{3}H]H_2DIDS also demonstrate the labelling of these same bands (Fig. 3). Moreover, this direct labelling in the isolated vesicles is correlated with the concentration-dependent inhibition of chloride-phosphate and lactate-lactate exchange by H_2DIDS. Treatment with 4 μM H_2DIDS strongly inhibits chloride-phosphate exchange, but leaves lactate-lactate exchange uninhibited; 4 μM [^{3}H]H_2DIDS labels predominately the band 3, along with a small amount of the 40–50 kDa band, in a ratio of 8 : 1. Treatment with 25 μM H_2DIDS inhibits the lactate-lactate exchange; 25 μM [^{3}H]H_2DIDS labels band 3 and the 40–50 kDa band in a ratio of 4 : 1. Finally, if the vesicles are first treated with 4 μM unlabelled H_2DIDS, then with 25 μM [^{3}H]H_2DIDS, the band 3 is not radiolabelled, but the 40–50 kDa band is. This result is very similar to what is reported for intact red blood cells [9,10]. Such results provide circumstantial but highly suggestive evidence that a band 3 polypeptide mediates inorganic anion transport and a 40–50 kDa polypeptide mediates lactate transport, both in intact cells [9,10] and vesicles. The ratio of counts in band 3 compared

to the 40–50 kDa band is about the same in vesicles labelled directly with 25 μM [^{3}H]H_2DIDS, and vesicles made from parent cells prelabelled with 25 μM [^{3}H]H_2DIDS, about 4 : 1. However, in the labelled parent cells before budding (Fig. 5a), this ratio is 7 : 1, which seems to indicate that an enrichment of the 40–50 kDa band relative to band 3 has occurred in the budded vesicles. This enrichment may be due to an actual relative increase in the amount of the putative lactate transporter. The 2-fold increase in the direct labelling of the 40–50 kDa band relative to band 3 by 25 μM [^{3}H]H_2DIDS compared to 4 μM [^{3}H]H_2DIDS, and the preferential labelling of the 40–50 kDa band by 25 μM [^{3}H]H_2DIDS following treatment with 4 μM H_2DIDS suggests that this labelling in the 40–50 kDa band is associated with the lactate transporter rather than some proteolytic fragment of band 3. Also, the major proteolytic fragment in unsealed rabbit ghosts treated with trypsin is not a 40–50 but a 50–60 kDa band (not shown), consistent with what is reported for human unsealed ghosts [32]. Also labelled in the vesicles treated directly with [^{3}H]H_2DIDS are 17 and 31 kDa dalton bands. These bands can be reduced in relative Coomassie-stained peak size and amount of radioactivity by treating the vesicles with a high ionic strength alkali solution (not shown), which indicates that they are probably not intrinsic membrane proteins. The labelling of thee bands is not observed in the vesicles made from prelabelled cells, which suggests that they are not proteolytic fragments of band 3 or the 40–50 kDa band. However, this does indicate that some peripheral or cytoplasmic protein (e.g., hemoglobin) is accessible to labelling by [^{3}H]H_2DIDS in the vesicles, but not in the intact cells. H_2DIDS is known to be impermeant in intact red cells [9]. If the vesicles are permeable to [^{3}H]H_2DIDS during the course of incubation, this compound would be expected to encounter and react with the large molar excess of intracellular hemoglobin.

Perhaps the best evidence that a particular protein actually mediates a transport process is to show that the isolated, solubilized protein performs the same process when reconstituted into a lipid bilayer. This has been accomplished for human band 3 [15–18], establishing directly that this protein mediates anion transport. Thus, the inference that the labelled band 3 polypeptide of vesicles mediates the observed chloride-phosphate exchange is supported by the precedent that a band 3 polypeptide from another mammalian red cell membrane functions as an anion exchanger. The reconstitution of the isolated lactate transporter has not been reported. Thus, the evidence that the labelled 40–50 kDa polypeptide is actually responsible for lactate exchange across the vesicles and the intact red cell [9,10] membranes is more circumstantial. This paper suggests that the functional lactate transporter is present in the vesicle membrane. These vesicles, which are quickly and easily prepared, may provide the starting material for future work in the isolation and reconstitution of the erythrocyte membrane protein responsible for lactate transport.

Acknowledgements

This work was supported by a grant from the U.S. National Institutes of Health (R01 GM 26861) held by Michael L. Jennings, in whose laboratory this work was carried out, and by a Medical Scientist Training Program Grant (GM 07337) from the U.S.N.I.H. I thank Dr. Jennings for his generous advice during the course of these experiments and in the preparation of the manuscript. I also thank Rick Roberts and Matt Theriac of the Central E.M. Facility and J.P. Wensel in the Department of Anatomy for providing electron micrographs of the vesicles.

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