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Reconstitution of glucose transport activity from erythrocyte membranes without detergent and its use in studying effects of ATP depletion

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The direct reconstitution of unsolubilized membrane proteins by the freeze-thaw procedure avoids possible changes in properties produced by detergent solubilization and fractionation. Glucose transport activity was reconstituted using human erythrocyte membranes, with about 2/3 of the glucose uptake being stereospecific. The highest specific activity occurred at low ratios of protein to lipid in the reconstitution, where most transport was due to liposomes containing single transporter molecules. Transporters were reconstituted with a scrambling of orientations, indicated by a 50% inactivation by added trypsin. Separation of unreconstituted protein doubled the specific activity. Similar results were obtained using the purified transporter (Wheeler, T.J. and Hinkle, P.C. (1981) J. Biol. Chem. 256, 8907–8914). The same ratio of net uptake to equilibrium exchange was observed for the two preparations. Their relative reconstituted transport activities and cytochalasin B binding activities were equal, indicating that the two were reconstituted with similar efficiencies. The decrease in glucose transport in erythrocytes produced by ATP depletion and the stimulation produced by resealing with ATP (Jacquez, J.A. (1983) Biochim. Biophys. Acta 727, 367–378) were confirmed. However, no difference was observed in reconstituted transport activity using ghosts resealed with or without ATP, indicating that ATP produces indirect effects rather than modifications of the transporter.

Introduction

Reconstitution of membrane transport activity in artificial membranes is now widely used in the characterization and purification of transport proteins. In typical reconstitution procedures (reviewed in Refs. 1 and 2), membrane proteins are solubilized in detergents and then reconstituted into liposomes or planar lipid bilayers. However, it is possible that the fractionation procedures used in purification and/or the detergent treatment alter the properties of the protein being studied.

The freeze-thaw reconstitution procedure [3] involves combining a membrane protein sample with preformed liposomes. The protein is usually contained within vesicles of endogenous lipid or lipid that has been added during the extraction and fractionation procedure; thus the method actually involves the fusion of two types of membranes. Since no detergent is involved in the freeze-thaw process itself, it is possible to use membranes that have never been exposed to detergent. This type of reconstitution differs from the typical reconstitutions in that the protein is never actually removed from a membrane. However, since the reconstituted liposomes will consist predominantly of

Abbreviations: Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; Mops, 4-morpholinepropanesulfonic acid; DMSO, dimethyl sulfoxide.

lipid derived from the preformed liposomes, the protein has in fact been transferred to a new membrane environment, which can be of defined composition and free of possible complicating factors in the intact cell. Thus one can properly speak of reconstituting the transport function in this new environment. Proteins reconstituted by this method will not have been subject to possible changes in their properties as a result of detergent treatment. The procedure may therefore be of use in the study of transport mechanism and regulation.

Franzusoff and Cirillo [4] reported that it was possible to reconstitute glucose transport activity in this manner using purified plasma membranes from *Saccharomyces cerevisiae* and liposomes of asolectin. The resulting liposomes were referred to as hybrid liposome–plasma membrane vesicles. During the course of our investigations of the reconstituted glucose transporters of human erythrocytes [5], bovine heart [6], and rat skeletal muscle (T.J. Wheeler and M.A. Hauck, unpublished experiments) we observed that it was possible to reconstitute glucose transport activity directly using membranes from these three sources that had not been extracted with detergent. In the experiments reported here glucose transport activity reconstituted from erythrocyte membranes is characterized. In addition it is shown how this technique can yield information on the regulation of the transporter and the extent to which its properties are preserved during extraction and purification. While this work was in progress, Weber et al. [7] reported studies that also include direct reconstitution of erythrocyte membranes using the freeze-thaw procedure.

Materials and Methods

Materials. Outdated human blood was a gift from the American Red Cross, Louisville, KY. Sources of radioisotopes and other chemicals were previously reported [5,6]. Erythrocyte ghosts were prepared by the procedure of Dodge et al. [8].

Reconstitution and assay of glucose transport activity. Ghost samples that had been frozen were sonicated for about 5 s in a bath-type sonicator before use. Preparation of liposomes, reconstitution, and glucose transport assays were as described [6], except that the amount of reconstituted liposomes used per assay was reduced to 20 μl (0.5 mg soybean lipid). The total intraliposomal volume, measured by the uptake of L-glucose in 24 h, was 1.1 and 1.3 μl in two experiments.

ATP depletion experiments. Fresh erythrocytes were washed, depleted of ATP, lysed, and resealed as described by Jacquez [9]. Glucose uptake assays using intact erythrocytes contained 50 μl resuspended cells at a hematocrit of 0.5 and 1 ml of an assay mixture containing 0.1 mM D-[^{14}C]glucose in balanced salt solution (60 mM NaCl/75 mM KCl/1 mM $MgCl_2$/10 mM Tris-HCl, pH 7.4). Assays were carried out in 13 × 100 mm test tubes. Solutions and test tubes were kept on ice except while mixing, which was done with a vortex mixer. Assays were terminated with 3 ml of a stopping solution containing 171 mM NaCl/0.1 mM phloretin/1.25 mM KI/1 μM $HgCl_2$/1% ethanol [10]. The tubes were centrifuged 15 s in a clinical centrifuge, the supernatant was removed, and the cells were washed with an additional 3 ml of stopping solution and centrifuged again. Washed cells were lysed in 3 ml H_2O. One aliquot of the lysate was used for determination of hemoglobin absorbance, allowing normalization of uptake data to the number of cells used. Another aliquot of 1.5 ml was treated with 0.3 ml 50% (w/v) trichloroacetic acid, centrifuged, and 1.0 ml was used for scintillation counting.

Assays of glucose uptake in resealed ghosts contained 50 μl ghost suspension (0.32 to 0.47 mg protein), 0.1 mM D-[^{14}C]glucose, and tracer L-[^{3}H]glucose in balanced salt solution in a total volume of 250 μl. Assays were carried out in 1.5 ml microcentrifuge tubes, with tubes and solutions kept on ice except while mixing. Assays were stopped with 400 μl of the above stopping solution and the tubes were centrifuged 1 min in a microcentrifuge. The ghost pellets were washed twice with 500 μl stopping solution and then transferred to vials for scintillation counting.

Other procedures. D-Glucose reversible cytochalasin B binding was measured using a microcentrifuge assay as described previously [6]; assays contained 15 μg protein. Protein was measured by the method of Lowry et al. [11] using 2% sodium dodecyl sulfate in all samples. For assays of pro-

tein in reconstituted liposomes before and after purification by centrifugation, the samples were first precipitated from a methanol/chloroform/water mixture as described by Wessel and Flügge [12]. Volumes used in this paper were doubled, and a total of 2 mg lipid was included in all samples. Phospholipid was measured using fluorescence enhancement of diphenylhexatriene [13].

Results

Time-course of glucose uptake

Liposomes prepared from crude soybean phospholipids were reconstituted with human erythrocyte membrane proteins by the freeze-thaw procedure, using ghosts that had not been treated with detergent. The time course of glucose utpake into the reconstituted liposomes is shown in Fig. 1. Uptake in this and other figures is expressed as equilibrated volume, or glucose space. The stereospecific uptake was 61–67% of the total over the 10 min period, and reached about half its maximum level between 1 and 2 min. This is much slower than was seen in studies using the purified transporter, which gave half-times for equilibration of less than 10 s [3,5] to 30 s [14]. The nonspecific uptake in this experiment (32 pmol/mg lipid in 1 min) falls between values of 16 [3] and 100 pmol/mg lipid in 1 min [5] reported for the purified transporter.

Effect of protein : lipid ratio in reconstitution

In our studies of the reconstituted bovine heart [6] and rat skeletal muscle transporters we observed that the specific activity of reconstituted glucose transport activity was very sensitive to the ratio of protein to lipid in the reconstitution. The highest specific activities were observed only at ratios below 6–8 μg protein/mg lipid for cholate-extracted membrane proteins and below 8–16 μg/mg for plasma membranes reconstituted without detergent. As the protein concentrations were increased beyond these ratios the total glucose uptake into the liposomes leveled off and then declined, indicating that other proteins present in the membranes interfered with the reconstitution and/or made the liposomes leaky. Fig. 2 shows results of a similar study with erythrocyte ghosts. The stereospecific equilibrated volume obtained at 2 min increased with increasing protein concentration throughout the range studied. However, the specific activity (expressed as μl/mg protein) was much lower at 20–40 μg protein/mg lipid than at 4–8 μg/ml, where a value of about 120 μl/mg was observed. Thus the protein level at which the

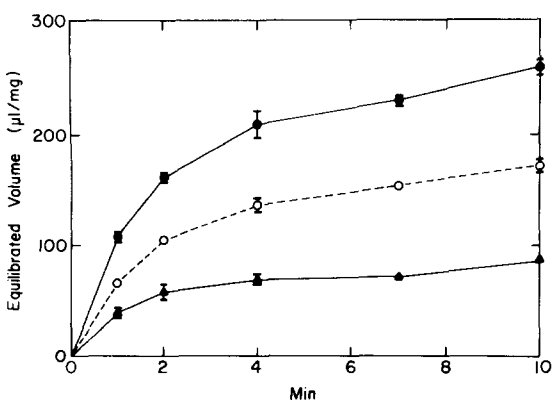

Fig. 1. Time-course of glucose uptake in liposomes reconstituted with unextracted human erythrocyte membrane proteins. Assays contained 2 μg protein, 0.5 mg soybean phospholipid, 0.2 mM D-[^{14}C]glucose, tracer L-[^{3}H]glucose, and 10 mM Mops buffer (pH 7.5) in a total volume of 0.25 ml. The uptake of glucose, expressed as equilibrated volume in μl per mg protein, is shown for D-glucose (●) and L-glucose (▲). The difference (D- minus L-glucose, ○) is the stereospecific uptake.

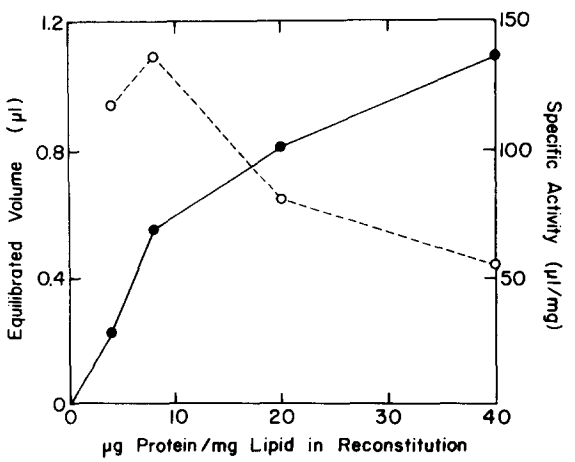

Fig. 2. Effects of protein concentration in reconstitution. Liposomes were reconstituted with 4–40 μg protein per mg soybean lipid. Stereospecific uptake (●) is expressed as μl equilibrated volume in assays containing 0.5 mg soybean lipid (left-hand scale). Specific activity is expressed as μl per mg protein (○) (right-hand scale). The total intraliposomal volume (determined in separate experiments) was about 1.2 μl.

specific activity bgins to be reduced is similar for the three types of membranes which we have studied.

It should be noted that the maximum equilibrated volume per mg protein (120 μl/mg in this experiment) is only about 2-fold lower than that reported for the purified glucose transporter reconstituted at low protein : lipid ratios (about 290 μl/mg, calculated from the data of Sase et al. [14]).

The question of whether the liposomes reconstituted with ghost proteins contain single or multiple glucose transporters was considered. Sase et al. [14], using a combination of kinetic results and freeze-fracture electron microscopy, showed that the purified transporter reconstituted by the freeze-thaw procedure was distributed randomly, independent of the size of the liposomes or the protein : lipid ratio in the reconstitution. A ratio of one transporter per liposome was achieved at about 5 μg protein per mg lipid. Since the ghosts used in the studies reported here had a specific activity of cytochalasin B binding (0.8 nmol/mg) about 7% of that of preparations of the purified transporter (11 nmol/mg [15]) about one transporter per liposome would be expected at a ratio of about 70 μg ghost protein per mg lipid, which is somewhat higher than was tested. Thus if the ghost proteins are also randomly distributed in the reconstitution, nearly all liposomes should contain only single transporters. Alternatively, it is possible that at increasing levels of protein there is a significant number of liposomes with multiple transporters. In that case the liposomes should equilibrate more rapidly, since uptake at early times is proportional to the number of transporters, while that at later points is limited by the intraliposomal volume (shown in Fig. 1 of Sase et al. [14]).

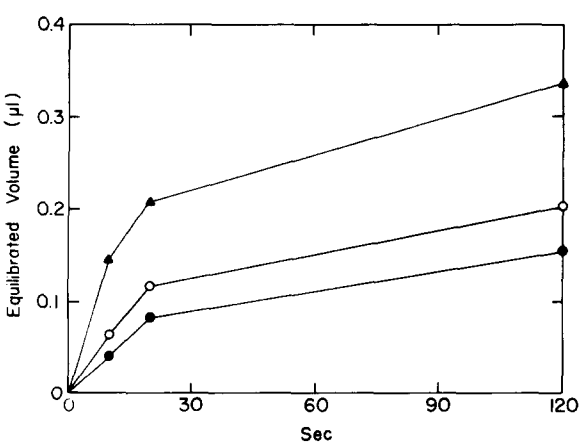

Fig. 3. Time-course of uptake in liposomes reconstituted at different protein : lipid ratios. Stereospecific glucose uptake was measured at 10, 20 and 120 s for liposomes reconstituted at 8 (●), 20 (○) and 40 (▲) μg protein per mg lipid.

Fig. 3 shows the uptake at 10, 20 and 120 s for liposomes reconstituted at 5, 10 and 20 μg protein/mg lipid. The fraction of the 2 min uptake seen at 10 s (26–31%) and 20 s (54–57%) was not significantly different for the two lower protein levels, but was somewhat higher (43%) at 10 s for 20 μg/mg. These results indicate that up to 20 μg/mg uptake is predominantly from liposomes with single transporters.

Effects of trypsin

Another question of interest is the orientation of the reconstituted transporter. Previously we used trypsin, which inactivates the transporter only from the cytoplasmic surface [16], to study the orientation of the purified transporter after reconstitution [5]. In these studies, externally added trypsin reduced the reconstituted activity about 40%, while trypsin present on both sides of the liposomal membrane inhibited about 80%. These results indicated that the protein was reconstituted with a random orientation.

The effects of trypsin treatment on the uptake of glucose in liposomes reconstituted at 4 and 20 μg protein/mg lipid were determined. The reconstituted liposomes were diluted to 50 or 250 μg protein/ml, respectively, and treated with trypsin (10 μg/ml) for 30 min or more. Uptake of 0.2 mM glucose in 10 and 120 s was determined. For the reconstitutions at 4 μg protein/mg lipid, trypsin inhibited 48 + 6% (mean ± S.E., $n = 3$) at 10 s and 38 ± 8% (4) at 2 min; for 20 μg/mg, the inhibition was 59 ± 12% (4) at 10 s and 33 ± 12% (4) at 2 min. These results support a random orientation for the transporter reconstituted from ghosts. They also support the idea that most uptake is due to liposomes with single transporters. If a liposome contained multiple transporters oriented randomly, trypsin would reduce its initial rate of uptake about 50%. However, it would not affect its final uptake as long as one or more

transporters were oriented as in the erythrocyte (and were therefore trypsin-insensitive). The 2 min uptake, a rough approximation of the final volume (Fig. 1), was reduced 33% by trypsin even at the higher protein level, indicating that about one-third of the liposomes containing transporters had no transporters with the right-side-out orientation.

Comparison of initial rates of uptake to intraliposomal volume

As noted above, uptake at early times (e.g., 10 s) reflects the activity of reconstituted transporters, while that at later times (e.g., 2 min) reflects the volume of liposomes containing active transporters, and thus the number of transporters. The ratio of these two uptakes is therefore a measure of the turnover number of active transporters. If the transporters were altered so that they retained partial activity, this should be reflected by a lower initial rate of uptake but a similar final volume compared to normal transporters. Fig. 4 plots the specific activities of initial rates of uptake (measured at 5 to 20 s) vs. equilibrated volume in 2 min for a number of experiments. In these experiments the reconstitutions were at 8 μg protein/mg lipid or less, where most transport appears to be in liposomes containing single transporters, as discussed above. The activities shown here varied over a 15-fold range as a result of such factors as variation in activity between ghost preparations, age of the preparations used, and the variability of the freeze-thaw reconstitution procedure. The data are consistent with a linear relationship between the two measurements; a fit by linear regression gave a correlation coefficient of 0.97. Thus over a wide range of activities the liposomes containing transporters equilibrate at similar rates. The variation appears to be primarily in the number of active transporters that have been reconstituted, rather than in their intrinsic activity; those transporters that transport to a significant level do so at similar rates.

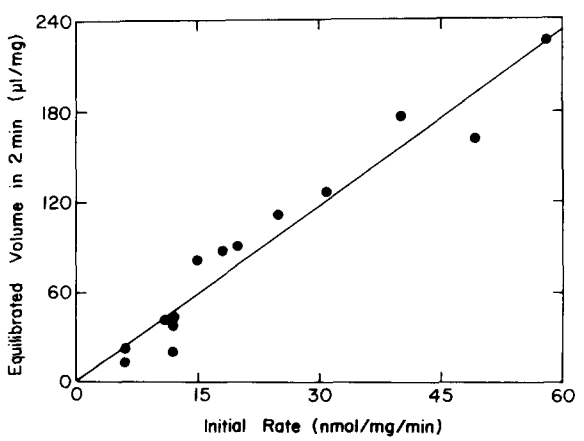

Fig. 4. Initial rates of uptake vs. equilibrated volume at 2 min. For 14 experiments using various preparations reconstituted at 2–8 μg protein/mg lipid, initial rate of stereospecific uptake (measured at 5–20 s) is plotted against the equilibrated volume measured at 2 min (representing from 2 to 40% of the total intraliposomal volume). The line was obtained from a fit of the data by linear regression.

Relative rates of equilibrium exchange and net flux

In our previous study of the purified transporter [5], we noted that the ratio of the V_{max} for exchange at equilibrium to that for zero-trans flux (30-fold) was higher than the ratios for erythrocytes (8–10-fold for uptake and 2–3-fold for efflux). Thus it was of interest to examine equilibrium exchange in the liposomes reconstituted with unextracted ghost proteins. Since most of the experiments reported here used net uptake of 0.2 mM glucose, the initial rate (measured at 20 s) of uptake at that concentration was compared to the initial rate of exchange of 20 mM glucose. In four such experiments, net uptake had an average rate of 19 nmol/mg per min, while exchange had a rate of 1.2 μmol/mg per min; the ratio (exchange/net) was 65 ± 5. In the experiments with the purified transporter, the initial rates under these conditions were 230 to 370 nmol/mg per min and 20 μmol/mg per min, respectively, giving a ratio of 54 to 87. Thus by this criterion equilibrium exchange compared to net flux is very similar for the two systems. However, a more complete comparison would require determination of the K_m and V_{max} values for net flux and equilibrium exchange using the reconstituted ghost proteins.

The above data also indicate that the purified transporter had about a 16-fold higher specific activity for both net flux and exchange compared to the ghost proteins. This is about the same as the ratio of specific activities of cytochalasin B binding (14-fold).

The equilibrated volume in 2 min was also compared for the conditions of net flux at 0.2 mM glucose and equilibrium exchange at 20 mM. The volume observed with exchange was only 43 ± 6% ($n = 4$) of that for net flux. This is in contrast to the results with the purified transporter, where essentially the same equilibrated volume was observed for both conditions. We did observe in the studies with the purified transporter that at concentrations above 40 mM a reduction in volume occurred, which was about 50% at 80 mM glucose. These results indicated that during the preequilibration period for the exchange experiments the liposomes shrink and do not regain their original volume. It appears that the liposomes reconstituted with the ghost proteins are more sensitive in this respect than those reconstituted with the purified transporter.

Fractionation of reconstituted liposomes by centrifugation

In our studies with the purified transporter [5] we observed that by layering reconstituted liposomes on 0.4 M sucrose followed by centrifugation at 25000 rpm for 30 min, a fraction of liposomes could be isolated which had a 2.5-fold higher specific activity than the starting reconstituted liposomes. This indicated that protein which had not been reconstituted was separated from the liposomes by the procedure. Similar experiments were performed using ghost membrane proteins reconstituted at 4 and 8 μg protein/mg lipid. Uptake of glucose at 10 s and 2 min was measured for the original reconstituted liposomes and from the fraction obtained from the sample–sucrose interface (the latter after removal of sucrose by gel filtration).

In two experiments, the ratios of specific activities (purified/original) were 2.6 ± 1.1 and 2.0 ± 1.2 at 10 s; and 1.8 ± 0.2 and 2.5 ± 0.5 at 2 min. Thus an increase in specific activity of about 2-fold was achieved by the fractionation, similar to the value obtained for the purified transporter.

Since the relative specific activities for the reconstituted purified transporter and ghost proteins are the same as their relative cytochalasin B binding activities, and the specific activity for transport for each is increased by about the same factor by separation of unreconstituted protein, the two types of samples appear to be reconstituted with similar efficiencies.

Effect of buffer used for liposome preparation

While the liposomes used in most experiments reported here were prepared by sonication of soybean lipids in 10 mM Tris buffer (pH 7.4), liposomes prepared in 10 mM Mops buffer (pH 7.4) were also tested occasionally. It was discovered that while Mops liposomes were capable of giving high reconstituted activity, more reproducible results were obtained with Tris liposomes. In six experiments where the two were compared directly, the Tris liposomes gave an average of 2.2 ± 0.6-fold higher activity (mean ± S.E.; range 1.0–4.7). A smaller effect was seen with reconstituted bovine heart plasma membrane extracts (1.6 ± 0.2-fold, $n = 4$), while in two experiments with the purified erythrocyte transporter no significant difference was seen. The cause of the different reconstitution efficiencies using the two types of liposomes is unknown.

Effects of ATP depletion on glucose transport activity

In order to demonstrate the application of the direct reconstitution procedure to studies of transport regulation, the effects of ATP depletion in erythrocytes on glucose transport activity [9] were studied. Jacquez observed a decrease in glucose transport activity when cells were depleted of ATP. Hemolysis and resealing with ATP or ADP reversed the effect. It was proposed that membrane protein phosphorylation was involved in the reversal.

In preliminary experiments, we observed that the mixing procedure employed by Jacquez was inadequate, and glucose uptake is not linear for 1.5 min at 4°C, as he reported, when a more vigorous mixing procedure using a vortex mixer (see Materials and Methods) is used. We initially considered that the results of ATP depletion could have been related to changes in glucose phosphorylation, which could affect the apparent rate of glucose accumulation in inadequately mixed cells. However, we confirmed the effect of ATP depletion on the transport rate using the vortex mixer procedure for both glucose and 3-*O*-methylglucose, ruling out this possibility. Also, we

TABLE I

EFFECTS OF ATP DEPLETION AND RESEALING OF GHOSTS WITH ATP

Fresh human erythrocytes were depleted of ATP by incubation with 20 μM $CaCl_2$ and 10 μg/ml A23187 in 1% DMSO for 1 h at 37°C. Control cells were kept on ice. Uptake of D-glucose in 10 s at 0°C was measured for the two sets of cells. The ATP-depleted cells were then lysed and resealed in the presence or absence of 1.6 mM ATP as described by Jacquez [9]. Stereospecific glucose uptake in 10 s at 0°C was determined for the resealed ghosts. The ghost proteins were then reconstituted at 4 and 8 μg protein/mg lipid for experiments I and II, respectively. Stereospecific glucose uptake in 10 s and 2 min at 20°C was measured.

	Relative glucose uptake	
	Expt. I	Expt. II
Erythrocytes:		
ATP depleted/control	0.49 ± 0.11	0.42 ± 0.05
Resealed ghosts:		
+ATP/−ATP	2.2 ± 0.4	4.6 ± 1.8
Reconstituted:		
+ATP/−ATP, 10 s	1.00 ± 0.33	0.95 ± 0.25
+ATP/−ATP, 2 min	1.13 ± 0.14	0.95 ± 0.16

observed about a 30% decrease in transport activity with cells incubated with 1% DMSO alone for 1 h at 37°C (compared to controls kept on ice), in contrast to the results of Jacquez, where no effect of incubation at 37°C with DMSO alone was observed. The decrease in activity produced by incubation at 37°C with DMSO alone was observed with both outdated and fresh blood.

In order to test whether the effect of ATP depletion on transport activity was an effect on the transporter itself, which might be expected to be preserved during reconstitution, the experiments described in Table I were performed. Fresh erythrocytes were depleted of ATP by incubation in the presence of calcium and the calcium ionophore A23187. Measurement of the initial rate of glucose uptake by the cells at 0°C revealed a 50–60% decrease in activity as a result of ATP depletion. The ATP-depleted cells were then hemolyzed and resealed in the presence or absence of 1.6 mM ATP. The initial rates of glucose uptake for the ghosts resealed with ATP were 2.2- and 4.6-fold higher than for those resealed without ATP. Finally, the resealed ghost proteins were reconstituted at 4 and 8 μg protein/mg lipid. No significant differences were observed for the reconstituted samples. These results argue against a modification of the transport protein itself and suggest that the results of different ATP levels and resealing may instead by indirect effects due to changes in the properties of the membrane.

Discussion

The direct reconstitution of unextracted membrane proteins by the freeze-thaw process avoids possible alterations in their properties as a result of detergent extraction. By comparing properties of the directly reconstituted proteins to those of proteins that have been extracted and purified, it may be possible to assess changes that have occurred during purification. The results described here show that the human erythrocyte glucose transporter is suitable for such a comparison.

The transporter reconstituted directly from erythrocyte membranes is similar to the purified, reconstituted transporter [5] in several respects: reconstitution in a scrambled orientation, as shown by trypsin inactivation; an increase in the specific activity of about 2-fold by removal of unreconstituted proteins during centrifugation; and a similar ratio of equilibrium exchange activity at 20 mM glucose to net uptake at 0.2 mM glucose. These results suggest that the transporter is not greatly altered during extraction and purification. Further experiments in comparing the kinetics would be of interest. For example, the reconstituted purified transporter showed a much higher ratio of equilibrium exchange parameters to net flux parameters than does the erythrocyte [5], which might be a result of purification of the protein or simply due to its different lipid environment. The origin of the asymmetry of glucose transport in erythrocytes is controversial, and it would also be of interest to investigate this phenomenon with reconstituted ghost proteins. Studies using the purified transporter after reconstitution and trypsin treatment indicated that the asymmetry was an inherent feature of the transporter protein [5].

The increase in specific activity of transport for the purified reconstituted transporter compared to the ghost proteins reconstituted directly is similar

to the increase in cytochalasin B binding activity. The simplest explanation of this is that the two activities are retained to similar degrees during extraction and purification, and that both samples are reconstituted with similar efficiencies. Racker [17] has warned that reliance on binding assays during membrane protein purification may lead to purification of a nonfunctional protein; however, in this case, cytochalasin B binding appears to be correlated with transport function.

The efficiency with which the transporter is reconstituted (which, as noted above, appears similar for the ghosts and for the purified transporter) can be estimated by combining kinetic measurements with measurements of equilibrated volume. In Ref. 5 we calculated a specific activity for the native transporter of 150 to 500 μmol/mg per min at 20 mM glucose. However, those figures should be doubled because the calculations assumed that the cytochalasin B binding sites corresponded to dimers; it now appears that a single 45 kDa polypeptide binds one molecule of cytochalasin B [18]. Therefore the specific activity of the purified reconstituted transporter (20 μmol/mg per min for samples not purified by centrifugation) [5] is about 2–7% of the native transporter. Using the data of Sase et al. [14], it can be calculated that under conditions where at most one transporter was reconstituted per liposome, the equilibrated volume was 290 μl/mg protein. However, using the diameter of 100 nm for liposomes having an average volume (Table I of Ref. 14), active transporters should equilibrate about 5300 μl/mg protein. The observed equilibrated volume is about 5% of this, which suggests that only about 5% of the transporters have been reconstituted in an active manner. Since the reconstituted transport rate is 2–7% of that for the native transporter, these results indicate that the transporter is reconstituted with low efficiency, but those transporters that are properly reconstituted have 40–100% of activity.

While several similarities between the reconstituted transport using ghost proteins and the purified transporter have been noted, minor differences were also observed. The liposomes reconstituted with ghost proteins equilibrated more slowly and were more sensitive to shrinking during incubation with 20 mM glucose than those reconstituted with the purified transporter. These differences might be due to a different size distribution of the two types of liposomes, but that has not been examined directly.

It is of interest to compare the results described here to those of Weber et al. [7], who also demonstrated reconstitution of erythrocyte glucose transport without detergent. The stereospecific equilibrated volume reported in Ref. 7 was about 65 μl/mg protein (calculated from data in Fig. 2), which is somewhat lower than reported here (120 μl/mg, Fig. 2). On the other hand, the equilibrium exchange rate calculated for 20 mM glucose (1.6–2.7 μmol/mg per min, using K_m = 25–30 mM and V_{max} = 4–6 μmol/mg per min) is higher than that reported here (1.2 μmol/mg per min). The reconstitution procedure used in Ref. 7 differed from that of this study in that acetone-washed lipids were used; a 100 mM KCl buffer was employed; and sonications were performed using a probe-type rather than a bath-type sonicator. Also, the protein : lipid ratio which was used (25 μg protein/mg lipid) was higher than the optimum seen here (10 μg/mg, Fig. 2).

The use of the direct reconstitution method was illustrated by studying the effects of ATP depletion on erythrocyte glucose transport [9]. Although ghosts resealed in the presence of ATP had much higher transport activity than those resealed in its absence, liposomes reconstituted with the two samples had the same transport activity. Since a covalent modification of the transporter, such as phosphorylation, might be expected to persist during the reconstitution, these results argue against such a modification. However, it is possible that an activity such as a phosphatase could reverse such a modification during reconstitution. Such a reversal would have to be relatively rapid, occurring after the ghosts are disrupted by the freeze-thaw step but before the sonication disperses transporters and modifying enzymes into separate liposomes. It might be possible to test whether phosphorylated transporters become dephosphorylated during the reconstitution; in vivo phosphorylation of the erythrocyte glucose transporter has recently been demonstrated [19]. However, in that study, the level of phosphorylation in the basal state was low and was increased substantially by a phorbol ester. This also argues against

the presence of ATP being necessary to maintain the transporter in a normally phosphorylated state.

It seems more likely that the effects of ATP are on the structure of the membrane, with resulting indirect effects on glucose transport. Depletion of ATP in erythrocytes is known to cause production of protuberances in the membrane. The effect is reversible and both it and its reversal can also be produced in ghosts [20]. Bursaux et al. [21] reported effects of ATP on erythrocyte anion transport similar to those on glucose transport. Thus the effects of ATP may be generalized effects on membrane phenomena rather than specific regulatory effects on transport proteins.

Reconstitution of membrane proteins without detergent extraction may be applicable to other transporters and other types of membrane proteins. In the experiments presented here, where a native membrane containing a high level of glucose transporters was employed, reconstituted transport activity was readily measurable and suitable for a variety of kinetic studies. When the membranes are not so greatly enriched, more difficulty will be encountered. For example, direct reconstitution of bovine heart plasma membranes, which have about 20-fold lower specific activity of glucose transporters, led to a reconstituted activity which was only about 1/2 stereospecific [6]. For other types of membrane proteins, such as ion-translating ATPases or receptor proteins, the problems of background activities may be much lower, and allow a variety of measurements using relatively unenriched membranes as a source.

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