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Isolation, Purification, and Reconstitution of a Proline Carrier Protein from *Mycobacterium phlei*†

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ABSTRACT: Membrane vesicles from *Mycobacterium phlei* contain carrier proteins for proline, glutamine, and glutamic acid. The transport of proline is Na^+ dependent and requires substrate oxidation. A proline carrier protein was solubilized from the membrane vesicles by treatment with cholate and Triton X-100. Electron microscopic observation of the detergent-treated membrane vesicles showed that they are closed structures. The detergent-extracted proteins were purified by means of sucrose density gradient centrifugation, followed by gel filtration and isoelectric focusing. A single protein with a molecular weight of 20 000 ± 1000 was found on polyacrylamide gel electrophoresis. Reconstitution of proline transport was demonstrated when the purified protein was incubated with the detergent-extracted membrane vesicles. This reconstituted transport system was specific for proline and required substrate oxidation and Na^+. The purified protein was also incorporated into liposomes, and proline uptake was demonstrated when energy was supplied as a membrane potential introduced by K^+ diffusion via valinomycin. The uptake of proline was Na^+ dependent and was inhibited by uncoupler or by sulfhydryl reagents.

Active transport of proline has been shown with the electron transport particles (ETP),[1] depleted ETP, cell membrane ghosts, and whole cells of *Mycobacterium phlei* (Hirata et al., 1971; Hirata & Brodie, 1972; Prasad et al., 1976). Proline transport in membrane vesicles exhibited a strict requirement for substrate oxidation as well as for Na^+ or Li^+ (Hirata et al., 1974b) and specific phospholipids (Prasad et al., 1975a). The uptake of proline proceeded against a concentration gradient with succinate, generated NADH, exogenous NADH, or ascorbate–*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TPD) as substrate. The transport of proline does not require a high-energy phosphate bond (Hinds & Brodie, 1974) since it proceeds in the absence of coupling factor or inorganic phosphate. It is inhibited by anaerobic conditions (Hirata et al., 1971). In addition to proline, the uptake of glutamine and glutamic acid has been demonstrated in the membrane vesicles from *M. phlei*, and it has been shown that the energy requirement and carrier protein(s) or binding site(s) involved in the uptake of glutamine and glutamic acid appeared to be different from those for the uptake of proline (Prasad et al., 1975b).

The active transport of metabolites across biological membranes has been studied in bacterial and mammalian systems. The energy source and the relationship between active transport and energy coupling have been investigated (Brodie et al., 1972; Kaback, 1974; Hirata et al., 1974a; Berger & Heppel, 1974) and reviewed (Simoni & Postma, 1975; Boyer & Klein, 1972; Harold, 1972; Kaback, 1972; Oxender, 1972). The existence of solute-specific membrane-associated proteins has been documented in bacterial systems for solute transport (Fox & Kennedy, 1965), solute binding (Piperno & Oxender, 1968), and group translocation (Kundig & Roseman, 1971a,b). Recently, carrier proteins, which may be defined as proteins which translocate solutes across the membrane, have been solubilized from bacterial and mammalian membranes by using detergents (Gordon et al., 1972; Kashara & Hinkle, 1976; Hirata et al., 1976; Shertzer & Racker, 1976; Crane et al., 1976; Amamuna et al., 1977; Lee et al., 1977a). The reconstitution of active transport with carrier proteins in-

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[1] Abbreviations used: ETP, electron transport particles; DETP, ETP depleted of the coupling factor latent ATPase; Tx-ETP, Triton X-100 treated ETP; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride.

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corporated into liposomes was demonstrated by utilizing a membrane potential driving force generated by K^+ diffusion via valinomycin (Hirata et al., 1976; Amanuma et al., 1977).

In this communication, we describe the solubilization of the proline carrier protein from the membrane vesicles of *M. phlei* and its purification and reconstitution. The purified protein restored Na^+- and substrate-dependent proline transport in the detergent-extracted membrane vesicles and reconstituted Na^+-dependent proline uptake in proteoliposomes. The driving force for the latter was supplied as a membrane potential induced by K^+ diffusion via valinomycin. A preliminary report of these studies has been reported (Lee et al., 1977a).

Experimental Procedures

Solubilization of Proline Carrier Protein. M. phlei (ATCC 354) was grown as described previously (Brodie & Gray, 1956), and the electron transport particles (ETP) were prepared by sonication of cells as described by Brodie (1959). The carrier protein was solubilized from ETP or coupling factor latent ATPase (BCF_1) depleted ETP (DETP) (Higashi et al., 1969, 1975) by treatment with cholate and Triton X-100. ETP or DETP was suspended in a solution of 0.5 M Tris-HCl buffer (pH 8.0) containing 1.0% sodium cholate and 0.15 M KCl at a protein concentration of 10 mg/mL. After incubation at 25 °C for 60 min, the mixture was centrifuged at 105000*g* for 45 min. The cholate-washed pellets were resuspended in the same volume of 50 mM Tris-HCl buffer (pH 8.0) containing 0.25 M sucrose, 0.15 M KCl, and 2.0% Triton X-100 by 2 min of sonication. They were then incubated for 30 min at 25 °C. The proline carrier protein was found in the supernatant after centrifugation of the Triton X-100 mixture at 144000*g* for 60 min. The detergent-treated membrane vesicles were found in the pellet and referred to as Tx-ETP. The Tx-ETP's were washed twice (144000*g* for 60 min) with 10 mM $MgCl_2$. The supernatant was concentrated in an Amicon Diaflo apparatus with a PM-10 membrane.

Purification of Proline Carrier Protein. The concentrated samples were partially purified by sucrose density gradient centrifugation in 0.7–1.0 M sucrose containing 0.15 M KCl at 175000*g* for 16 h in a SW 50.1 rotor by using a Spinco L-2 ultracentrifuge (Beckman Instruments). The gradient was removed by puncturing the bottom of the centrifuge tube. Six fractions were obtained as described previously (Lee et al., 1976), and each fraction was assayed for the ability to restore proline transport with Tx-ETP. The carrier protein was found in a dark yellow band at a density of approximately 0.8 M sucrose (fraction III). As shown in Figure 1, this fraction was almost free of Triton X-100 since most of the detergent remained on the top of the sucrose gradient. Fraction III was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 0.25% sodium cholate for 16 h at 4 °C. The sample was chromatographed on a Sephadex G-100 column (2.5 × 30 cm) which was previously equilibrated with the same buffer at 4 °C. The column was eluted with the same buffer and fractions of 5 mL were collected. The cytochromes were eluted in the void volume. All later fractions containing proteins were pooled, concentrated, and dialyzed against water for isoelectric focusing. Spectral analysis at this stage of the purification showed that the proteins contained no detectable electron transport components.

Isoelectric Focusing. Carrier ampholytes were synthesized by following the method of Vesterberg (1969). In trial runs a 4% solution of these ampholytes established a broad pH range (2.5–10.5) after isoelectric focusing. A Kontes Chromaflex column (1.0 × 30 cm) with a cooling jacket was packed with deaerated Sephadex G-10 and equilibrated with

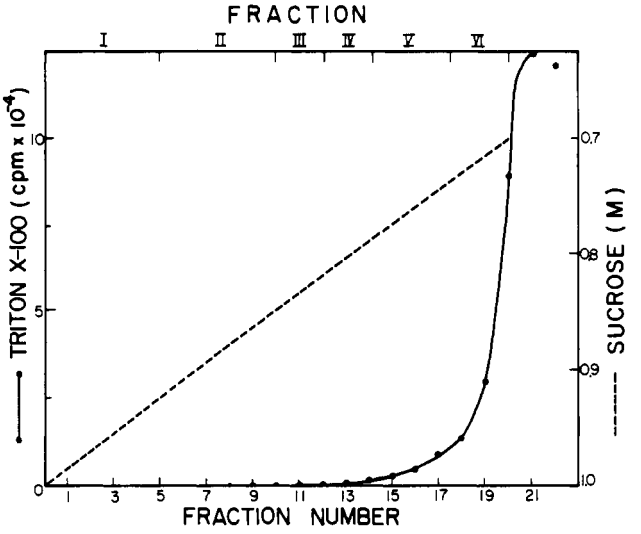

FIGURE 1: Distribution of Triton X-100 on a sucrose density gradient centrifugation. The crude Triton X-100 extract (0.6 mL containing 1.0 μCi/mL [*phenyl*-^{3}H]Triton X-100) was layered over a 0.7–1.0 M linear sucrose density gradient containing 0.15 M KCl. The tubes were centrifuged in a Spinco SW 50.1 rotor at 175000*g* for 16.5 h in a Spinco L-2 centrifuge. Fractions (0.25 mL) were collected by puncturing the bottom of the tube. The radioactivity in the fractions was counted in a Nuclear Chicago liquid scintillation detector in Bray's solution.

a 4% solution of ampholytes containing 10% glycerol. After application of the samples to the column, additional ampholyte solution was added until the protein band was near the center of the column. The top of the column was sealed with a 10% polyacrylamide gel. The column was inverted and the column stopper removed from the new top of the column. Phosphoric acid solution (10%) was gently layered on the top, and the bottom was placed in a beaker of 10% ethanolamine, with care taken to remove all trapped air below the column. The cooling bath (4 °C) was then connected. The positive electrode was connected to the top (acid) and the negative electrode attached to the bottom (base). The column was then focused at 400 V for 18 h. Bands could be clearly distinguished. After disconnection, the stopper was inserted and the column was again inverted. Fractions of 0.4 mL each were collected with a microfractionator (Gilson Medical Electronics).

Gel Electrophoresis. Disc gel electrophoresis was performed on 7.5, 10, and 12.5% polyacrylamide gels in a Tris–glycine buffer, pH 8.3 (Davis, 1964). Protein on the gel was stained with 0.05% Coomassie blue. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate for the determination of molecular weight was carried out by the method of Weber & Osborn (1969).

Measurement of Proline Binding. An ultrafiltration method was used to measure the binding of proline (Lee et al., 1977b), using ultrafiltration cells (MRA Corp., Boston, MA) with PM-10 membranes (Amicon).

Assay of Amino Acid Transport in Tx-ETP. The washed, detergent-treated vesicles (Tx-ETP) were suspended in 1.5 mL of reaction mixture containing 50 mM Hepes–KOH (pH 7.0), 10 mM $MgCl_2$, 10 mM NaCl, and carrier proteins as indicated. After 10 min of preincubation at 30 °C, the reaction was started by the addition of 25 μM proline (containing [^{14}C]proline, 1.0 μCi/mL) and ascorbate–TPD (17 and 1.5 μM, respectively) as substrate. At indicated time intervals, 0.1-mL aliquots were removed and immediately diluted in 2.0 mL of 50 mM Hepes–KOH buffer (pH 7.0). The suspension was rapidly filtered on a membrane filter (Millipore, 0.45 μm), and the filters were washed twice with the same buffer. The

filters were rapidly removed and dried, and the radioactivity retained on the filters was counted in a Nuclear Chicago liquid scintillation detector by using a scintillation fluor with Beckman Bio-Solv (BBS-3).

Preparation of Proteoliposomes. Soybean phospholipids (azolectin from Associate Concentrates), partially purified by acetone extraction (Kagawa & Racker, 1971), were suspended in 0.2 mM potassium phosphate (pH 8.0) containing 0.3 M KCl and sonicated for 10 min in a 10Kc Raytheon sonic oscillator at 1.0 A (100% output) at 2 °C under N_2 gas to give a suspension of 50 mg/mL. The proteoliposomes were prepared by combining the phospholipid suspension with the same volume of the purified carrier protein and then sonicating for an additional 30 s under N_2 gas. The mixture was centrifuged at 166000*g* for 10 min by using a Beckman airfuge (Beckman Instruments). The K^+-loaded vesicles were suspended in 1 mL of 0.1 mM potassium phosphate (pH 8.0) containing 0.15 M KCl and assayed for proline uptake. Under these conditions about 50% of the carrier protein was incorporated into the liposomes.

Assay of Proline Uptake in Proteoliposomes. The K^+-loaded proteoliposomes were suspended in 1.0 mL of reaction mixture containing 50 mM Hepes–KOH (pH 7.5), 10 mM $MgSO_4$, and 10 mM NaCl. After 3 min of preincubation at 30 °C, proline (final concentration 25 μM containing [^{14}C]proline, 1.0 μCi/mL) was added. After 2 min of further incubation, the reaction was started by the addition of valinomycin at a final concentration of 1 μM. At the indicated time intervals, 0.1-mL aliquots of the mixture were removed, filtered (Millipore, 0.22 μm), washed, and assayed as described above.

Analytical Procedures. Phospholipids in the carrier protein preparation were extracted by the method of Folch et al. (1951), and phospholipid phosphorus was determined according to the method of King (1932). Protein concentration was determined by the method of Lowry et al. (1951) or a modification of the Biuret method (Dawson et al., 1969), with bovine serum albumin as standard.

Materials. Sodium cholate, Triton X-100, valinomycin, and all of the standard proteins for molecular weight determination were purchased from Sigma Chemical Co. [^{14}C]Proline, [^{14}C]glutamine, [^{14}C]glutamic acid, [*phenyl*-^{3}H]Triton X-100, [*carbonyl*-^{14}C]dextran, and ^3H_2O were from New England Nuclear Corp. All other chemicals were of reagent grade purity.

Results

Intactness of Detergent-Treated Membrane Vesicles. Electron microscopic observations were made of Tx-ETP following negative staining with 2% phosphotungstic acid. Although many of the membrane vesicles were irregularly shaped, they appeared to be intact and devoid of coupling factor ATPase structure (Asano et al., 1973).

More convincing evidence for sealed membrane vesicles has been provided by the observation, under phase-contrast microscopy, that vesicles were found to shrink when the osmolarity of the medium was increased. As shown in Figure 2, the Tx-ETP and regular ETP both showed a marked response to changes in osmolarity, as observed by measuring the changes in light scattering in different concentrations of sucrose. This indicated that the vesicles were closed structures. A similar effect that was due to changes in osmolarity was found in *Escherichia coli* membranes (Kaback & Deuel, 1969).

Further studies with the proline uptake and intravesicular volume demonstrated that both have a similar response to the effect of osmolarity. The intravesicular volume of membrane vesicles was estimated from the difference between the total ^3H_2O-permeable space and [*carbonyl*-^{14}C]dextran-impermeable space (Waddell & Butler, 1959; Hunter & Brierley, 1969). The intravesicular volume of Tx-ETP was reduced from the original volume by 53% by the addition of sucrose at a final concentration of 1 M, while that of the regular ETP was reduced by 62%. Proline transport was also reduced approximately 50–60% by the addition of sucrose to a final concentration of 1 M.

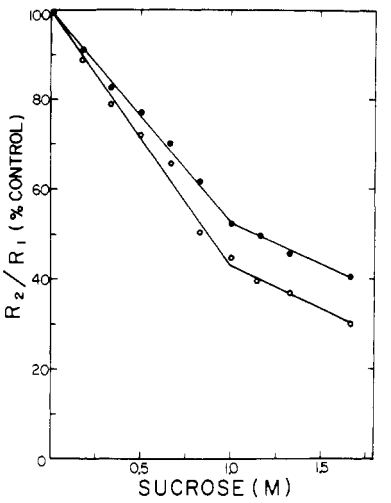

FIGURE 2: Effect of increasing sucrose concentration on light scattering properties of membrane vesicles. Approximately 3 mg of regular ETP or Tx-ETP was suspended in 3.0 mL of 0.15 M KCl containing various concentrations of sucrose as indicated in the figure. The samples were equilibrated at room temperature for 15 min and scatter/absorbance ratios (R_2/R_1) were then measured at 720 nm by using a MPF-4 Perkin-Elmer spectrofluorometer at R_2 = 90° and a Gary 14 spectrophotometer at R_1 = 180°. (●) Regular ETP; (○) Tx-ETP.

Restoration of Proline Transport in Tx-ETP. Each fraction obtained from the isoelectric focusing column was assayed for restoration of proline transport with Tx-ETP as well as for proline binding activity (Figure 3). Fractions 13–17 (peak B) restored proline transport, and this activity was specific for proline. Amino acid binding activity (proline, glutamine, and glutamic acid) was found in fractions 3–6 (peak A). It should be noted that peak B also bound proline, but to a lesser extent than peak A (based on protein content). Similarly, although a negligible amount of transport activity was restored by fractions in peak A, this was much less than that observed with peak B. These results suggested that the binding and carrier proteins for proline transport may be different. For further studies on the carrier protein, the active fractions in peaks A and B were pooled separately and dialyzed against 50 mM Tris-HCl buffer, pH 8.0, followed by several changes of deionized water to remove ampholytes.

Disc Gel Electrophoresis. The purity of the proline carrier protein obtained from the isoelectric focusing column was ascertained by gel electrophoresis. On 7.5 and 10% polyacrylamide gels at pH 8.3, only one protein band was observed (Figure 4). After gel electrophoresis with sodium dodecyl sulfate, there was also a single band. Thus, there appears to be just one polypeptide in the carrier protein (Figure 4D).

The molecular weight of the purified carrier protein was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 0.1% $NaDodSO_4$ and 10% acrylamide. A plot of log molecular weight of standard proteins vs. the mobility was linear (data not shown). From the comparison of the mobility of the carrier protein band with those of standard proteins of known molecular weight, a polypeptide

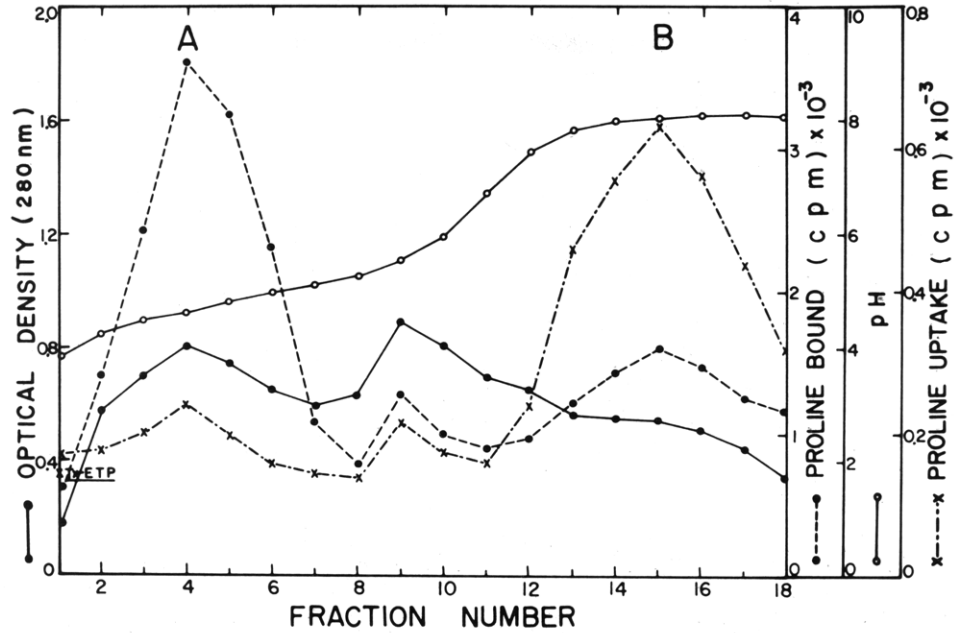

FIGURE 3: Isoelectric focusing of proline binding and carrier proteins. The column was prepared and the sample (1.5 mL containing 3.2 mg of protein) was applied as described under Experimental Procedures. Protein was estimated by spectrophotometric absorbance at 280 nm, and pH was measured with a Corning Digital 112 Research pH meter. Binding of proline was determined by the ultrafiltration method (see Experimental Procedures) by using a reaction mixture containing 0.1 mL of the fraction, 50 mM Hepes–KOH (pH 7.5), 10 mM $MgCl_2$, 10 mM NaCl, and 25 μM proline containing 1.0 μCi/mL [^{14}C]proline in a final volume of 0.5 mL. The incubation was carried out for 30 min at 30 °C with shaking, and 0.2-mL aliquots were filtered and counted. Proline transport was assayed as described under Experimental Procedures, by using 1.0 g/mL Tx-ETP and 0.1 mL of the fractions.

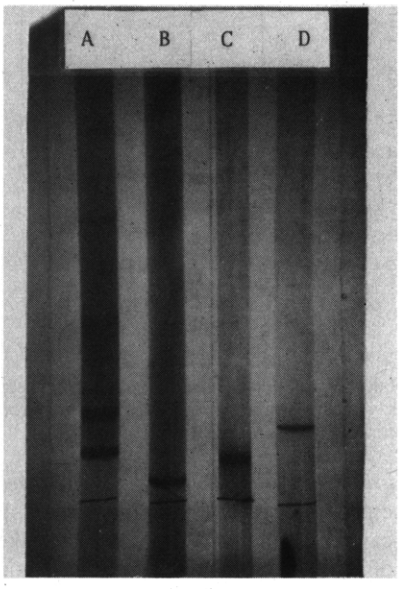

FIGURE 4: Polyacrylamide disc gel electrophoresis of the proline carrier protein. Gels were run at room temperature as described under Experimental Procedures. (A) Sephadex G-100 fractions, 10% gel; (B) isoelectric focusing fractions (peak B), 7.5% gel; (C) isoelectric focusing fractions (peak B), 10% gel; (D) isoelectric fractions (peak B), 10% gel with 0.1% sodium dodecyl sulfate.

Table I: Purification of Proline Carrier Protein[a]

fractions	protein added (mg)	proline transport (pmol/min/mg of protein)	total protein recovery (mg)
membrane vesicles (DETP)			1000.0
cholate extract	1.00	1	83.2
Triton X-100 extract	1.00	6	180.5
sucrose density (fraction III)	0.50	25	18.9
Sephadex G-100	0.10	73	6.0
isoelectric focusing (peak B)	0.01	1327	2.3

[a] Transport was assayed as described under Experimental Procedures by using Tx-ETP (1.5 mg of protein) and different fractions as indicated above. The reaction was carried out at 30 °C for 15 min. Transport is expressed as picomoles per minute per milligram of proline carrier protein.

molecular weight of 19 000–21 000 was calculated. This does not exclude the possibility that the active protein may be oligomeric.

Restoration of Proline Transport in Tx-ETP as a Function of the Amount of Carrier Protein Added. The ability of the purified carrier protein to restore proline transport with detergent-treated membrane vesicles from *M. phlei* is shown in Table I. Although some transport of proline was observed in the Tx-ETP alone (58 pmol/mg of protein), the addition of the purified carrier protein resulted in a four- to fivefold stimulation of transport. For determination of the concentration gradient, the intravesicular volume of Tx-ETP was estimated. The intravesicular volume was found to be approximately 2.1 μL/mg of protein, and the reconstituted Tx-ETP established a concentration gradient of proline of about fivefold when ascorbate–TPD was used as substrate. The proline transport in the reconstituted Tx-ETP was increased by increasing the amounts of purified carrier protein. As shown in Figure 5, the level of proline uptake was nearly proportional to the amount of carrier protein added over the range of 0–15 μg with 1 mg of protein of Tx-ETP.

The uptake of proline reached a steady-state level after 15–20 min of incubation at 30 °C (Figure 5 inset). In contrast to reconstituted ETP, regular ETP reached a steady-state level of proline transport after 5–10 min (Hirata et al., 1974b). The longer time required by the reconstituted Tx-ETP's may be due to their lower oxidative capabilities (Lee et al., 1976).

Specificity of Proline Transport in Reconstituted Tx-ETP. The reconstitution of proline transport by the carrier protein

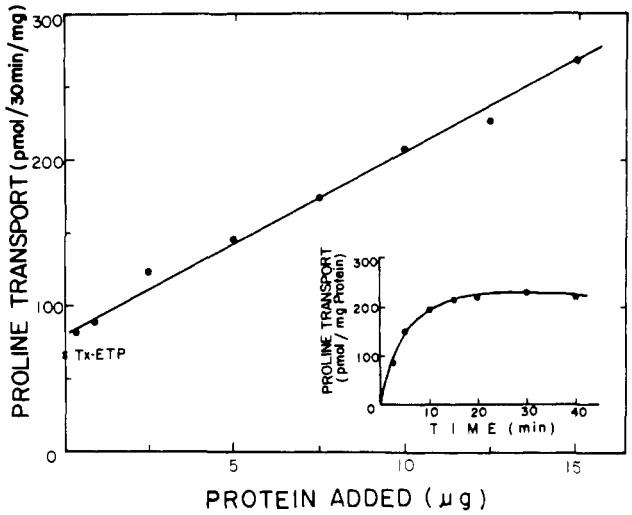

FIGURE 5: Proline uptake by Tx-ETP reconstituted with various amounts of carrier protein. The reaction mixture was similar to that described in Table I, by using 1.0 mg of protein of Tx-ETP and 1–15 μg of carrier protein. The insert shows the time course of proline uptake into reconstituted Tx-ETP by using 10 μg of carrier protein per mg of Tx-ETP.

Table II: Reconstitution of Amino Acid Transport in Tx-ETP[a]

	transport of amino acids (pmol/mg of protein)		
	proline	glutamine	glutamic acid
Tx-ETP	61	131	150
Tx-ETP plus CP	233	137	161

[a] The reaction mixture was similar to that described under Experimental Procedures using 1.5 mg of Tx-ETP and 10 mg of purified carrier protein (CP) obtained from isoelectric focusing of peak B. Total volume was 1.5 mL, and the reaction was carried out at 30 °C for 15 min. Glutamine and glutamic acid were added to a final concentration of 25 μM containing 1.0 μCi/mL [^{14}C]-glutamine or [^{14}C]glutamic acid.

(peak B) was shown to be specific for proline. As shown in Table II, the addition of carrier protein (peak B) did not stimulate the uptake of glutamine and glutamic acid into Tx-ETP. The combination of peak A and peak B did not increase the level of proline transport over that observed with peak B alone (data not shown). Other hydrophobic proteins such as bovine serum albumin were inactive. Furthermore, the addition of other membrane proteins such as the BCF_0–F_1 complex (DCCD-sensitive latent ATPase) or BCF_0 (intrinsic membrane protein) did not affect the restoration of the proline transport (data not shown).

The purified carrier contained 0.024 mg of phospholipid per mg of protein. The addition of phospholipids such as azolectin or lipids extracted from *M. phlei* membranes had no effect on the reconstitution of proline transport into Tx-ETP described in the present study.

Kinetics of Proline Transport in Reconstituted Membrane Vesicles. Studies were carried out to determine whether the proline transport system in the reconstituted membrane vesicles had an altered affinity for proline compared to the untreated membrane vesicles. The apparent K_m for proline transport in electron transport particles and in the reconstituted membrane vesicles was determined from Lineweaver–Burk plots (Figure 6). The plots were linear, and the apparent K_m was found to be the same (6.25 μM) in both the untreated ETP and the reconstituted Tx-ETP. Thus, the affinity of the transport system for proline in reconstituted membrane vesicles was not altered.

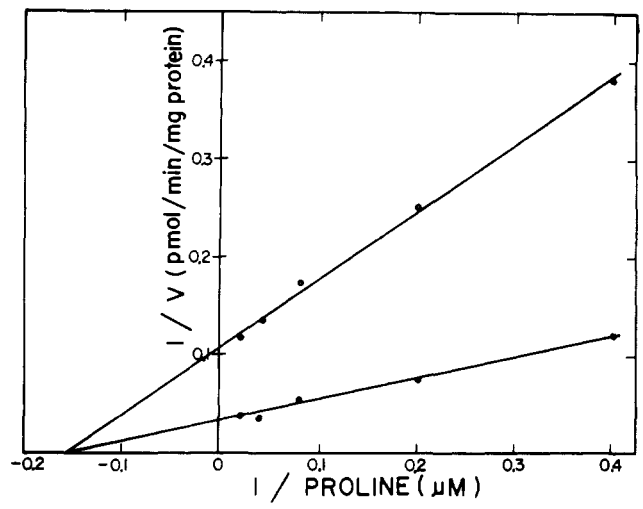

FIGURE 6: Lineweaver–Burk plots for proline transport. Purified carrier protein (10 μg/mg of Tx-ETP) was used to reconstitute the Tx-ETP. Experimental conditions were similar to those described under Experimental Procedures. (Upper line) Reconstituted Tx-ETP; (Lower line) regular ETP.

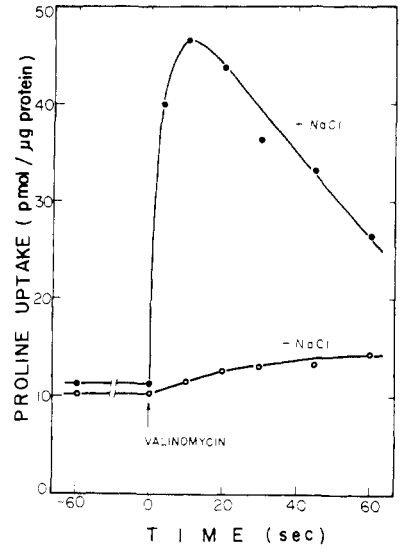

FIGURE 7: Uptake of proline by reconstituted proteoliposomes. Proline uptake was assayed as described under Experimental Procedures. (●) Complete system; (○) complete system minus NaCl.

Reconstitution of Proline Uptake in a Proteoliposomal System. The proteoliposomes containing entrapped K^+ and the purified carrier protein showed a rapid uptake of proline on addition of valinomycin and Na^+. The maximal uptake of proline was reached about 10 s after the addition of valinomycin (Figure 7). This uptake was specific for proline; glutamine and glutamic acid were not transported. Proline uptake into the proteoliposomes required the presence of Na^+.

In proteoliposomes prepared from various amounts of the purified carrier protein and a fixed amount of phospholipids (5 mg of azolectin), the initial rates of proline uptake were nearly proportional to the amount of protein over the range of 1–10 μg (data not shown). With concentrations over 10 μg, the uptake of proline reached a plateau, suggesting that the maximal level of transport may occur at a ratio of phospholipids to carrier protein of 500 to 1. Phospholipids extracted from *M. phlei* are equally as good for the reconstitution of proline transport when proteoliposomes are prepared as described under Experimental Procedures. It should be noted that the stoichiometry with respect to the amount of carrier protein and restoration of proline uptake with the

Table III: Effect of Inhibitors on Proline Uptake by Reconstituted Proteoliposomes[a]

compounds added	proline uptake: nmol/mg of protein	proline uptake: % of inhibn
none	48.6	0
carbonyl cyanide *m*-chlorophenylhydrazone (0.01 mM)	12.6	74
dicyclohexylcarbodiimide (0.6 mM)	50.1	0
N-ethylmaleimide (1 mM)	18.8	61
p-(chloromercuri)benzenesulfonic acid (1 mM)	14.3	70

[a] The reaction mixture was similar to that described in Figure 6. The inhibitors were preincubated with the proteoliposomes in the reaction mixture at the indicated concentrations for 10 min at 30 °C. The reaction was started by the addition of valinomycin at a final concentration of 1 μM. Incubations were carried out for 10 s at 30 °C.

liposomal system was better than that observed with the Tx-ETP. This inconsistency may be related to the nature of rebinding of the purified protein to the Tx-ETP.

The uptake of proline induced by valinomycin was inhibited by a proton conducting uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone, but was not inhibited by dicyclohexylcarbodiimide (Table III). It was also inhibited by sulfhydryl reagents such as *N*-ethylmaleimide or *p*-(chloromercuri)benzenesulfonic acid.

Discussion

The carrier protein for proline transport was solubilized from the membrane vesicles of *M. phlei* in order to gain some information concerning the bioenergetic requirements for active transport of amino acids. The carrier protein solubilized by treatment of membrane vesicles with cholate and Triton X-100 was extensively purified. This preparation was capable of proline transport when added to detergent-treated membrane vesicles (Tx-ETP) or to phospholipid vesicles. Detergent treatment of membrane vesicles from *M. phlei* resulted in a loss in the cytochrome content and in a lowering of the extent of substrate oxidation (Lee et al., 1976). Furthermore, the ability to couple phosphorylation to substrate oxidation was almost completely lost in the Tx-ETP. The addition of purified BCF_0–F_1 complex (DCCD-sensitive coupling factor latent ATPase) increased the rate of oxidation slightly and restored coupled phosphorylation with Tx-ETP (Lee et al., 1976). Detergent treatment of DETP produced similar observations to that described for Tx-ETP. This system is useful for the resolution and reconstitution of the membrane components involved in oxidative phosphorylation as well as in active transport of proline.

Detergent treatment of ETP destroyed the capability for active transport of proline. However, the addition of purified carrier protein restored proline transport, and it was further observed that the proline transport was nearly proportional to the amount of the carrier protein added with a constant amount of Tx-ETP protein. Proline transport in these reconstituted Tx-ETP's was specific for proline, was Na^+ dependent, and required substrate oxidation. It is pertinent to mention that other fractions obtained from the isoelectric focusing column also exhibited a high level of proline binding activity. Although fractions in peak A exhibited a greater level of proline binding activity, only fractions in peak B were capable of restoring transport activity (Figure 3). These results may suggest that the binding and the carrier proteins for proline are different. However, the binding capacity for proline in peak A was found to be nonspecific. Further study on the binding and carrier proteins for other amino acids such as glutamine and glutamic acid is therefore required. Although the original membrane vesicles from *M. phlei* transport proline, glutamine, and glutamic acid, only the proline carrier protein was solubilized under the conditions described in this report. The protein (peak A) which displayed nonspecific binding activity for these three amino acids produced a much lower level of transport in the reconstituted system than that observed with peak B.

The hydrophobic carrier protein was easily incorporated into the Tx-ETP or Tx-DETP, since the membrane contained about 30–50 μg of Triton X-100 per mg of protein after being washed twice with 10 mM $MgCl_2$ or water. This was estimated by using [*phenyl*-^{3}H]Triton X-100. The Tx-ETP also contained 23.7 μg of phospholipid per mg of protein, whereas 59.7 μg of phospholipid was estimated from regular ETP. It is pertinent to determine whether the reconstitution of proline uptake with detergent-treated membrane vesicles was due to a specific carrier protein or was merely a reflection of making the vesicles less leaky. Mizushima (1976) has reported that detergent-treated membrane vesicles from *E. coli* were almost completely deficient in proline uptake but that the addition of a large amount of bovine serum albumin resulted in the restoration of the proline transport activity. It was concluded that the restoration of the transport was due to removal of the detergents which were bound to the *E. coli* membrane vesicles (Mizushima, 1976). In the present study, however, the addition of bovine serum albumin or other hydrophobic proteins isolated from *M. phlei* did not affect the uptake of proline into Tx-ETP over the range of 0.005–0.5%.

The kinetic data revealed that the K_m for proline in the restored transport system was the same as that observed for proline in the untreated membrane vesicles from *M. phlei*, indicating that the affinity for proline in reconstituted Tx-ETP was not altered. Phospholipase A treated DETP's have been shown to have a lower steady-state level of proline transport compared with untreated DETP, but results of kinetic studies indicated that the decreased level of proline transport was not due to a change in the apparent K_m for proline (Prasad et al., 1975a).

Proline uptake was reconstituted in phospholipid vesicles with purified carrier protein by using a membrane potential introduced by K^+ diffusion via valinomycin. The uptake of proline into these proteoliposomes occurred within 10 s after the addition of valinomycin in the presence of Na^+. A similar reconstitution of amino acid transport has been demonstrated by Hirata et al. (1976) and Amanuma et al. (1977). The reconstitution of proline transport in the proteoliposomes displayed properties similar to those observed with the original membrane vesicles and the reconstituted Tx-ETP, including a requirement for Na^+. A requirement for Na^+ or the stimulation by Na^+ of active transport of amino acids and various substrates has been reported in a number of bacterial (Stock & Roseman, 1971; Halpern et al., 1973; Miner & Frank, 1974; Lanyi et al., 1976; Tsuchiya et al., 1977) and eucaryotic systems (Wheeler & Christensen, 1967;]967; Murer & Hopfer, 1874; Lever, 1977). It has been shown that a proton gradient is formed on the addition of ascorbate and benzoquinone to a preparation of proteoliposomes in which ferricyanide was entrapped inside the liposomes as an electron acceptor. The addition of Na^+ and proline then resulted in the inward movement of Na^+ and proline, with a simultaneous collapse of the proton gradient (Lee & Brodie, 1978).

Proline uptake by reconstituted vesicles was inhibited by sulfhydryl reagents. It was previously shown that sulfhydryl

groups are involved in both the oxidation of substrates and the active transport of proline by membrane vesicles from *M. phlei* (Kosmakas & Brodie, 1974). A cupric ion mediated uptake of proline in membrane vesicles of *M. phlei* has been described, which depends on the oxidation of a sulfhydryl group of either the carrier or some other membrane protein(s) by Cu^{2+} (Jacobs et al., 1978).

Recent work has indicated that preparation of liposomes by sonication, as in the studies described here, produces multilamellar vesicles (Tyrrell et al., 1976). These would be unsuitable for transport studies in which information on the concentration gradients established was required. For the purposes used here, however, i.e., identification of the capabilities of the isolated carrier protein to support the transport of proline with requirements similar to those of the original membrane vesicles, the sonicated liposomal preparations provide a model system for studying the mechanism(s) of active transport of amino acids.

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Quaternary Structure and Arrangement of Subunits in Hemocyanin from the Scorpion *Leirus quinquestriatus*†

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ABSTRACT: A study of the quaternary structure of hemocyanin from the scorpion *Leirus quinquestriatus* was carried out. The amino acid composition and the copper content were determined. For the native molecule, a sedimentation coefficient of 36.2 S and a molecular weight of 1.74×10^6 were found. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis, five bands were obtained. Most of the material migrated with a mobility corresponding to a molecular weight of 74×10^3. Dissociation of the molecule at alkaline or acidic pH yielded a 5S component corresponding to individual polypeptide chains. At the transition pH values, intermediate products of dissociation with sedimentation coefficients of 15.4 and 23.9 S, attributed to quarter- and half-molecules respectively, were obtained. Electron micrographs of the native molecule showed two structures, a rectangle and two rhombi diametrically connected together at the corners of the obtuse angle. We propose a model for the molecule consisting of 24 spherical subunits, each subunit representing a 5S particle. Every six subunits are organized in an octahedral arrangement representing a 16S unit, and the 36S molecule is a square-planar structure composed of four 16S units. The square arrangement of the 16S units is consistent with an analysis of the hydrodynamic data by the Kirkwood treatment, and projections of the model agree with particle profiles observed in the electron micrographs. Implications of the proposed structure on the observed heterogeneity of monomeric 5S subunits are discussed.

Hemocyanin is a copper containing respiratory protein found in the hemolymph of molluscs and arthropods. Since the early work of Svedberg and co-workers (Eriksson-Quensel & Svedberg, 1936), it has been realized that the hemocyanins can exist in a number of discrete states of aggregation—100, 60, 20, and 11 S for molluscan and 60, 36, 24, and 16 S for arthropod hemocyanins (Van Holde & Van Bruggen, 1971). Electron microscopy (Mellema & Klug, 1972; Siezen & Van Bruggen, 1974; Van Breemen et al., 1977) has provided what is now a generally accepted model for the assembly of molluscan hemocyanins. Much progress has also been achieved in the elucidation of the structure of hemocyanins from arthropod origin (Van Bruggen, 1968; Van Holde & Van Bruggen, 1971; Lontie & Witters, 1973; Antonini & Chiancone, 1977). Here, ultracentrifugation and electron microscopy have established the 16S aggregation state as the basic structural building block. Electron microscopy has provided, in addition, information on the spatial arrangement of the 16S units in the oligomeric structures. However, the structure of the 16S unit, by itself, is still unresolved. Although it has been established beyond doubt that the 16S unit is a hexamer of 5S subunits—polypeptide chains of about 75 000 daltons that constitute the ultimate dissociation product of all arthropod hemocyanins—the assembly of these subunits remains controversial. No less than five different models have been proposed (Andrews & Jeffrey, 1976).

Hemocyanin from the scorpion *Leirus quinquestriatus* is currently under study in our laboratory. Some of its spectral and functional properties have already been described (Klarman et al., 1977; Klarman & Daniel, 1977). The purpose of the present report is to present the results of a structural study carried out on this arthropod hemocyanin.

Materials and Methods

Preparation of Hemocyanin. Hemocyanin from the scorpion *L. quinquestriatus* was prepared as previously described (Klarman et al., 1977). Unless otherwise stated, hemocyanin solutions were made in buffers devoid of Ca^{2+} and Mg^{2+}. Hemocyanin concentrations were obtained from absorbance measurements at 280 nm. The extinction coefficient at this wavelength was determined by measuring the number of Rayleigh fringes produced in a synthetic boundary experiment in the ultracentrifuge by a sample of known absorbance and taking a value 4.08 fringes/(mg/mL) determined for hemocyanin from *Busycon* (Quitter et al., 1978).

Amino Acid and Copper Analysis. Protein samples were hydrolyzed with constant boiling HCl in sealed and evacuated Pyrex ampules at 110 °C for 24, 48, and 72 h. The amino acid composition was determined by ion-exchange chromatography in a Beckman Unichrom amino acid analyzer (Spackman et al., 1958). Tryptophanyl to tyrosyl ratio was determined spectrophotometrically by the method of Edelhoch (1967). Half-cystine and cysteine were determined as cysteic acid according to Hirs (1956). Copper content was determined by atomic absorption using a Varian Model AA5 spectrophotometer.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate gel electrophoresis was performed in vertical gel slabs (7.5% acrylamide, 0.2% bis(acryloylamido)methane, 0.035% *N,N,N′,N′*-tetramethylenediamine, 0.03% ammonium peroxodisulfate), using a Hoefer Scientific Instruments apparatus, Model SE 500. Samples for electrophoresis were incubated in Tris-HCl buffer containing 2% sodium dodecyl sulfate and 1% 2-mercaptoethanol for 2 min at 100 °C. Tris–glycine

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