A Hydrophobic Protein from the Cell Envelope of *Hydrogenomonas facilis**

Harry G. Rittenhouse, John Heptinstall,† and Bruce A. McFadden‡

ABSTRACT: A phospholipoprotein precipitates when the ionic strength of the "soluble" fraction from *Hydrogenomonas facilis* is lowered. The derived protein, which electrofocuses into a single sharp peak at pH 9 in 10 M urea, is extremely hydrophobic. Sedimentation to equilibrium in 8 M guanidine hydrochloride reveals an apparent molecular weight of 31,350 ± 500. The carboxymethylated protein or phospholipoprotein gives a single protein peak (mol wt 31,000–34,000) upon elution from Sephadex G-75 with 8 M urea in 25% formic acid. The minimum molecular weight measured by electrophoresis in polyacrylamide gel containing 10% sodium dodecyl sulfate is 15,000. The sole amino-terminal residue detected by dansylation is alanine. An immune response has been obtained to the phospholipoprotein as evidenced by a single precipitin band after double diffusion or immunoelectrophoresis in agar containing 1% Triton and 1 M urea. Using antiserum to the phospholipoprotein, precipitin lines of identity are obtained between the following: phospholipoprotein, the derived protein, dissolved spheroplast envelope, and the dissolved envelope fraction. The soluble fraction gives four precipitin bands one of which is probably identical with that for phospholipoprotein. Agglutination of *H. facilis* by antiserum to the phospholipoprotein establishes that the antigen is on the exterior surface of the cell wall.

Numerous enzymes and electron-transfer components are known to be associated with the bacterial cytoplasmic membrane (Marr, 1960). It was therefore not surprising that many protein components were observed by Schnaitman (1970a) in *Escherichia coli* derived cytoplasmic membranes. Of particular interest, however, was the added observation that 70% of the cell wall protein contained component(s) of a molecular weight of 44,000. Accordingly Schnaitman suggested that this major constituent of the cell wall was a structural protein. In light of the controversy over the occurrence of structural protein in eukaryotic organisms (Ashwell and Work, 1970), the isolation of possibly primitive archetypes from bacteria assumes particular significance. Part of the controversy stems from indications that some eukaryotic structural protein may be denatured ATPase (Senior and MacLennan, 1970). Thus isolation under mild conditions is important to minimize this possibility.

Kuehn *et al.* (1969) recovered a precipitate of semiordered structure upon lowering the ionic strength of the high-speed supernatant derived from extracts of *Hydrogenomonas facilis*. The precipitate, a phospholipoprotein, contained a strongly aggregating protein of unknown cellular origin that showed a marked resemblance in composition to "structural proteins" from eukaryotic sources. We now describe the further characterization of this protein.

Materials and Methods

Materials. Ultra Pure urea and guanidine hydrochloride from Mann Research Laboratories were used without further purification. SDS[1] was obtained from Matheson Coleman & Bell, Freunds adjuvant from Difco Labs., Triton X-100 from Sigma Chemical Co., and Ampholine carriers from LKB Instruments, Inc. Sephadex and Agarose were obtained from Pharmacia Fine Chemicals and coomassie brilliant blue from Mann Research Labs. The following proteins of high purity were obtained from (1) Mann Research Laboratories, catalase; (2) Calbiochem, cytochrome *c;* (3) Worthington Biochemical Corp., chymotrypsin; (4) Sigma Chemical Co., aldolase, chymotrypsinogen, lysozyme, pepsin, ribonuclease, and trypsin.

Methods. The phospholipoprotein was recovered by lowering the ionic strength of the 100,000*g* supernatant derived from mid- to late-log-phase cells of *H. facilis* (Kuehn *et al.*, 1969). The precipitate contains about 70% phospholipid, 30% protein, and less than 0.01% sugars (Kuehn *et al.*, 1969). To remove phospholipid prior to isoelectric focusing the complex was sonicated in the presence of chloroethanol. The protein was then precipitated with $CHCl_3$–1-butanol (3:1, v/v) and extracted consecutively with: (1) 1-butanol, (2) CH_3OH, (3) $CHCl_3$–1-butanol–CH_3OH (1:1:1, v/v), (4) CH_3OH, and (5) water. It was then dissolved in 10 M urea, and electrofocused for 1 hr in polyacrylamide gel polymerized chemically from 10% acrylamide containing 10 M urea (Wrigley, 1968). The upper and lower vessels contained 5% ethanolamine and 5% H_3PO_4, respectively.

Prior to sedimentation studies a sample of phospholipoprotein dissolved in chloroethanol was either: (a) heated to 95° for 15 min, cooled, and precipitated with $CHCl_3$–1-butanol (3:1, v/v), and washed with CH_3OH and water or (b) chromatographed upon Sephadex LH-20 using chloroethanol for elution followed by removal of chloroethanol *in vacuo*. The protein was then dissolved in 8.0 M guanidine·HCl and dialyzed against nine volumes of this solvent. Sedimentation equilibrium studies were then conducted and the data analyzed by computer as described earlier (Kuehn

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† Present address: Department of Biochemistry, University of Leicester, Leicester, England.

‡ The author to whom inquiries should be sent.

[1] Abbreviations used are: sodium dodecyl sulfate (SDS); 1-dimethylaminonaphthalene-5-sulfonyl (dansyl).

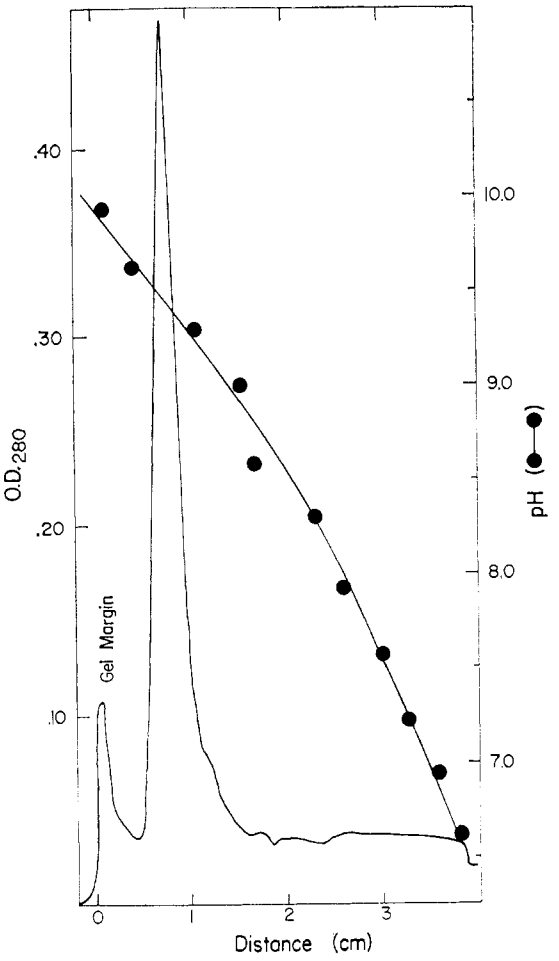

FIGURE 1: Isoelectric focusing of the protein derived from phospholipoprotein.

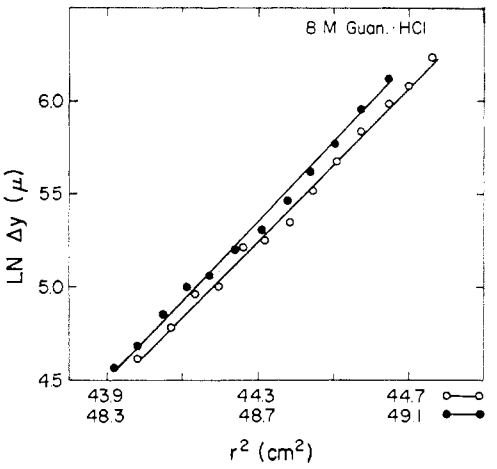

FIGURE 2: Plots of equilibrium distribution of the protein after sedimentation at 48,000 rpm for 23 hr at 20°. (●,○) Proteins after removal of phospholipid by methods (a) and (b), respectively.

and McFadden, 1969). The partial specific volume of the protein of 0.73 was estimated from the amino acid composition (Kuehn *et al.*, 1969, and Kuehn and McFadden, 1969).

For molecular weight measurements using gel filtration, the general procedure of Fish *et al.* (1969) was used with Sephadex G-75 except that the eluting solvent was 8 M urea in 25% formic acid. For measurements using polyacrylamide gel electrophoresis, the method of Weber and Osborn (1969) was employed except that 10% SDS replaced 0.1% SDS and 20% sulfosalicylic acid was used to elute the SDS prior to staining. Coomassie brilliant blue was used to detect bands in gels (Weber and Osborn, 1969).

For detection of amino-terminal residue(s), the protein dissolved in 10 M urea was treated with dansyl chloride and hydrolyzed as described by Gray (1967) except that a 6.5-hr hydrolysis time was used. Thin-layer chromatography was used for identification of dansylamino acids using solvent systems A, B, C, and D of Morse and Horecker (1966).

An immune response was obtained by injecting rabbits subcutaneously with 6 mg of phospholipoprotein dissolved in 0.2 ml of complete Freunds adjuvant every 4 days for 2 weeks. After a primary response had been detected, the animals were bled and the antibodies concentrated by precipitation at 0° with $(NH_4)_2SO_4$ added to 50% saturation. The nature of the immune response in all cases was identical when antigen had been injected in physiological saline. Immunodiffusion and immunoelectrophoresis at pH 8.0 were conducted in 1.5% Agarose containing 1% Triton X-100 and 1 M urea (Demus and Mehl, 1970).

Spheroplasts were prepared by treatment of late-log-phase *H. facilis* with lysozyme and EDTA at pH 8.0 (Costerton *et al.*, 1967). After lysis, fragments were collected by centrifugation onto 2 M sucrose (Schnaitman, 1970a). The interface band was then washed four times with 50 mM Tris, pH 7.5 (25°), containing 10 mM EDTA and finally dissolved in the Triton–urea solvent. Alternatively, a suspension of whole spheroplasts in 0.5 M sucrose containing 50 mM Tris, pH 8.0 (25°), was tested for agglutination by antiserum to phospholipoprotein as described below.

In tests for agglutination of intact bacterial cells by antiserum to phospholipoprotein, one volume of a suspension of a late-log-phase culture (*ca.* 10^9 cells/ml) in 0.9% NaCl, was incubated at 40° for 2 hr with one volume of the specified dilutions of antiserum.

The crude envelope fraction was recovered from cell-free preparations as that fraction sedimenting between 3000*g* and 100,000*g* (Schnaitman, 1970a).

Results

Figure 1 shows that the protein derived from the phospholipoprotein electrofocused in a single sharp band in 10 M urea at an apparent pH of 9.4. If a correction is made for the minor but significant increase in the measured pH by high concentrations of urea (Ui, 1971) the apparent pI becomes 9 indicating that about 5 of 23 residues of Asp and Glu in the hydrolysate (Kuehn *et al.*, 1969) are present as the corresponding amide. The polarity index, p (Fisher, 1964), calculated by assuming that 18 residues are evenly divided between Asp and Glu is 1.01 for V_t of 9348 Å^3 suggesting that the protein should be very strongly aggregating.

In Figure 2, plots of data are displayed for meniscus-depletion sedimentation equilibrium studies in 8 M guanidine·HCl. The lack of fringe displacement in the top half of the solution column (not shown) plus linearity of the plots are compatible with homogeneity of the protein. The molecular weights, uncorrected for preferential interaction of solute with third component, are 32,200 ± 500 and 30,500 ± 500 for protein samples from which phospholipid had been removed by the two procedures described.

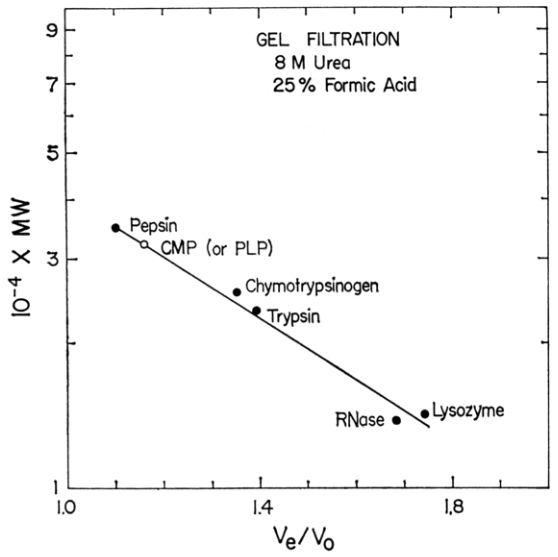

FIGURE 3: Semilog plot of molecular weight (MW) *vs.* elution from gel for the phospholipoprotein (PLP), the carboxymethylated protein (CMP) derived from PLP, and standards at ambient temperature.

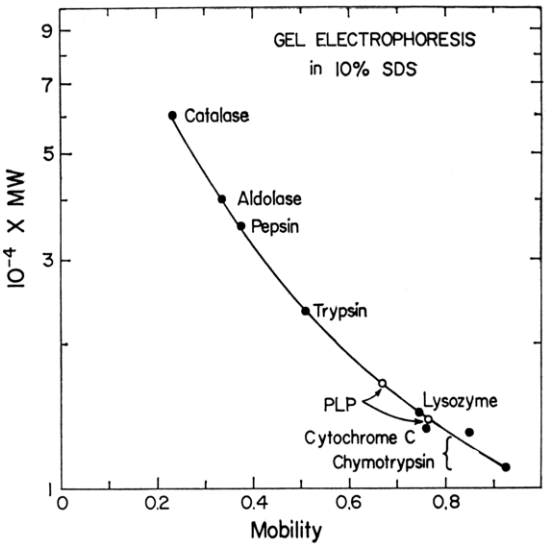

FIGURE 4: Semilog plot of MW *vs.* mobility of the phospholipoprotein and standards after electrophoresis of the phospholipoprotein (PLP) at ambient temperature in gel containing 10% SDS. Although the two extreme MW's for PLP are shown, two other independent MW measurements fell within this range.

Filtration by Sephadex G-75 using 8 M urea–25% formic acid as a solvent reveals a single species of protein in the phospholipoprotein or the carboxymethylated protein (Crestfield *et al.*, 1963) derived from it. Molecular weights were in the range of 31,000–34,000 for four samples and only the average is shown (Figure 3).

Polyacrylamide gel electrophoresis of the phospholipoprotein in 0.1% SDS originally revealed the presence of several protein species of molecular weights of *ca.* 15×10^3, 30×10^3, 45×10^3, and 60×10^3. Elution of each of these proteins and subsequent gel electrophoresis revealed the same multiple-band pattern once again. Gel electrophoretic studies in 10% SDS were ultimately successful in dissociating the strongly aggregating protein to essentially a single component of molecular weight in the range of 14×10^3 to 16.5×10^3. The migration of two different samples is shown in Figure 4.

Only alanine could be detected as an amino-terminal residue by the technique of dansylation. Phospholipoprotein contains no pigment and has no detectable Na^+- or Mg^{2+}-activated ATPase activity (Rockstein and Herron, 1951).

In Figure 5A, the presence of two components in the 100,000*g* supernatant mother liquor that show immunological cross-reaction with phospholipoprotein is established by double diffusion. One shows identity with phospholipoprotein. In Figure 5B a spheroplast envelope component that shows immunological identity with phospholipoprotein is revealed; also it is established that bovine serum albumin and denatured bovine serum albumin do not precipitate with antibody to phospholipoprotein. Sera from noninjected animals do not give precipitin with phospholipoprotein. High-speed supernatant denatured for 2 hr at 95°, does not give precipitin with antiserum to phospholipoprotein. Both protein and phosphate-free, carboxymethylated protein derived from phospholipoprotein show immunological identity with phospholipoprotein establishing that the immune response is to the protein component. No precipitin is obtained when the antiserum is diffused against culture filtrate from late log-phase cells of *H. facilis*. Thus, the phospholipoprotein is not excreted into the medium in contrast to a lipopolysaccharide–phospholipoprotein complex excreted during growth of *Escherichia coli* (Rothfield and Pearlman-Kothencz, 1969).

In Figure 6A, the greater resolving power of immunoelectrophoresis at pH 8 reveals the presence of four cross-reacting components present in the 100,000*g* supernatant (*cf.* Figure 5A), one of which moves toward the anode. One of the faster components moving toward the cathode may be identical with the phospholipoprotein whose migration is shown in Figure 6B. These four components are seen in the

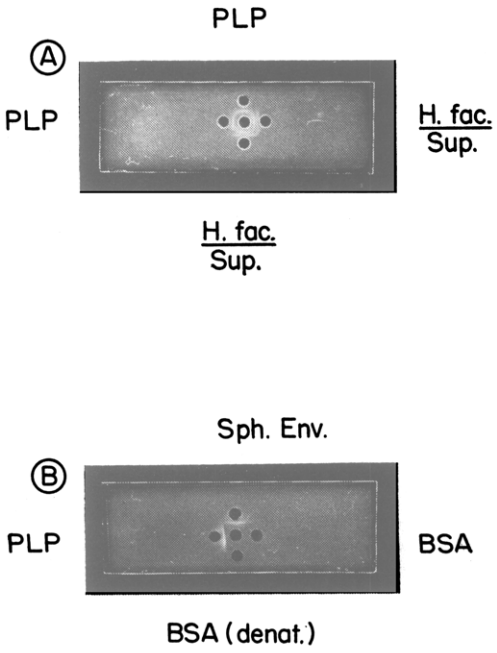

FIGURE 5: (A) Photograph representing double-diffusion studies of phospholipoprotein (PLP) and 100,000*g* supernatant (Sup) *vs.* antiserum (center well) to PLP. (B) Analogous to part A, showing, in addition, antiserum to PLP *vs.* BSA, denatured (denat.) BSA, and spheroplast envelope (Sph. Env.).

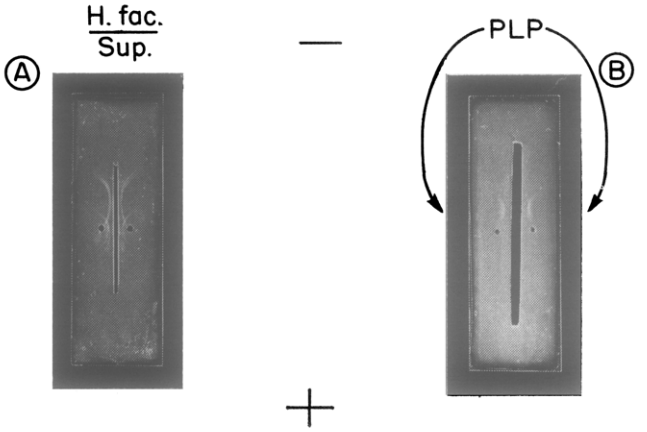

FIGURE 6: (A) Photograph representing immunoelectrophoresis of the 100,000*g* *H. facilis* (*H. fac.*) supernatant (Sup) originally in the wells *vs.* antibody to PLP in the trench. (B) Analogous to part A with PLP originally in the wells.

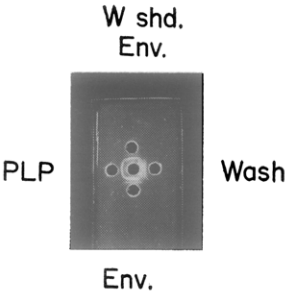

FIGURE 7: Photograph representing double-diffusion studies of PLP, the unwashed cell envelope fraction (Env.), washed envelope (Wshd. Env.) derived from *H. facilis*, and the fluid that had been used to wash the envelope (Wash).

supernatant regardless of whether phospholipoprotein has been precipitated.

When the 100,000*g* supernatant is treated (1) for 60 min at 25° with two volumes of antiserum followed by 12-hr incubation at 2°, and (2) the precipitate removed and washed two times with water, and procedures 1 and 2 are repeated three times, all four of the components giving precipitins in immunoelectrophoresis can be removed. The pooled precipitins are then fractionated by releasing the antibody into solution at 25° for 48 hr at pH 2.0 (0.01 N HCl–1% NaCl). The insoluble residual components, which are free of γ-globulin by the criterion of gel electrophoresis, comprise 9% by weight of the original soluble protein. The amino acid composition is shown in Table I and is significantly different from that of the phospholipoprotein.

In Figure 7, it is established by double diffusion that the crude cell envelope of *H. facilis* contains one component showing immunological identity with phospholipoprotein and one other component yielding a faint precipitin band. The latter can be removed by a single washing with 50 mM Tris, pH 7.8 (25°), containing 1 mM EDTA and detected in the wash fluid. It may be derived from the cytoplasm. The other component yielding the strong precipitation line cannot be removed by ten consecutive washes under the same conditions. Unfortunately, the techniques used by Schnaitman (1970a) to separate cell walls and cytoplasmic membranes from the envelope fraction of *E. coli* yield several ill-defined fractions when applied to cell envelopes from *H. facilis*.

In Figure 8, agglutination of intact cells of *H. facilis* by varying dilutions of the antiserum to phospholipoprotein is depicted. No agglutination is evident in the absence of antiserum (C_1), in the presence of anti-bovine serum albumin (C_2), or with serum obtained from untreated rabbits (not shown). Although not shown, suspensions of spheroplasts from *H. facilis* are also agglutinated by the antiserum. Cells treated for 1.5 min at highest power with a Virtis 23 mixer (Virtis Instruments, Inc.) to remove pili and flagella are still agglutinated.

TABLE I: Comparison of Amino Acid Composition of High-Speed Supernatant Components Recovered from Precipitins[a] with That of Phospholipoprotein.

Amino Acid	Components from Precipitins	Protein from Phospholipoprotein[b]
Lys	0.59	0.66
His	0.16	0.23
Arg	0.47	0.78
Asp	(1.00)	(1.00)
Thr	0.89	0.56
Ser	0.74	0.48
Glu	1.19	1.08
Pro		0.48
Gly	1.06	0.87
Ala	1.04	1.18
Cys		0.19
Val	0.97	0.75
Met		0.13
Ile	1.29	0.52
Leu	1.16	1.08
Tyr	0.27	0.22
Phe	0.32	0.34

[a] Protein was hydrolyzed for 48 hr at 105° with 6.7 N HCl and the composition determined with a Beckman 121 automatic amino acid analyzer. [b] Data from Kuehn *et al.* (1969).

Discussion

The present work indicates that the protein of a phospholipoprotein which precipitates under mild conditions from extracts of *H. facilis* is homogeneous. It is extremely hydrophobic having an apparent minimum molecular weight of 15,000 and readily forms oligomers even in the presence of

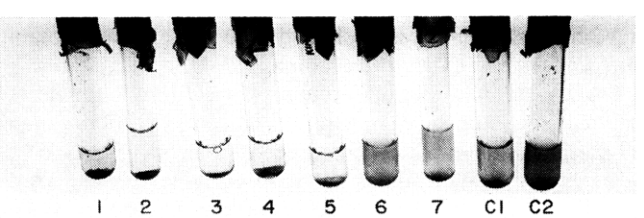

FIGURE 8: Photograph showing agglutination of *H. facilis* by the following dilutions of antiserum to phospholipoprotein: undiluted (1), 1–5 (2), 1–25 (3), 1–125 (4), 1–625 (5), 1–3125 (6), 1–15,625 (7). C_1 and C_2 represent incubations of *H. facilis* cells in the absence of the antiserum and in the presence of anti-bovine serum albumin, respectively.

dissociating agents such as 8 M urea–25% formic acid, 0.1% SDS, or 8 M guanidine hydrochloride. Indeed in earlier work it was established that multiple peaks obtained after gel electrophoresis of this protein in 5 M urea–35% acetic acid had relative mobilities characteristic of oligomers and identical amino acid compositions (Kuehn *et al.*, 1969). The hydrophobicity and very basic properties (pI of *ca.* 9) of this protein perhaps account for its strong association with phospholipid. These properties suggest that it is derived from the cell envelope.

Of interest is the presence of four immunologically cross-reactive species in the mother liquor from which the phospholipoprotein has been separated. Although one shows identity with the protein present in phospholipoprotein, the other three have different charge:mass ratios; one is acidic. The four proteins together have a different amino acid composition from that of the antigenic protein. Thus, although these components share antigenic determinants with phospholipoprotein they are not oligomeric. The state and relationship of these supernatant components to the phospholipoprotein and the cell wall protein await further clarification. The components with which the cell wall protein is associated must also be identified.

The present work establishes that the protein from *H. facilis* is, in part at least, on the exterior surface of the cell wall since whole cells can be agglutinated by antiserum to the phospholipoprotein. The observed agglutination also reduces the possibility that the phospholipoprotein is simply a denatured enzyme. The detection of a possibly more aggregated but analogous protein in cell walls of several gram-negative bacteria (Schnaitman, 1970b) may be of deep comparative biochemical significance.

Experiments are in progress to ascertain the function, exact locus, and biosynthetic origin of the envelope protein from *H. facilis* and the natural distribution of related proteins.

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