amino residues of the Hin peptide, is not sufficient to elicit specific binding to DNA. There may be additional nucleotide recognition elements within the COOH-terminal 52–amino acid residue sequence.

These experiments demonstrate the usefulness of solid matrix peptide synthesis for studying structure-function relations in proteins. It is especially noteworthy that the 52mer binds DNA without the need for purification after synthesis. Peptides, such as the 52mer and analogs that incorporate specific reporter groups, will provide powerful tools for further dissecting precise details of DNA protein interactions.

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20. These experiments were done with a ^{35}S or ^{32}P end-labeled DNA restriction fragment that contains both *hix*L and *hix*R separated by a 299-bp piece of DNA containing the recombinational enhancer. The construction of this fragment, which comes from pMFB36, will be described elsewhere (*17*). Hin was prepared as described earlier (*7*, *15*). Protection reactions were in a volume of 50 μl and the standard buffer contained 20 m*M* tris-HCl, *p*H 7.6, 100 m*M* NaCl, 5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, and polycytidylic acid (100 μg/ml). DNase I was incubated for 30 seconds at 0.4 μg/ml. For DMS experiments sodium cacodylate replaced the tris-HCl and dithiothreitol was omitted, and DMS was used at 85 m*M* for 30 seconds. Samples with high concentrations of either the 52mer or the 31mer were often poorly soluble in formamide. This problem was minimized when samples were first digested with proteinase K at *p*H 9 in 2*M* $NH_4CH_3CO_2$, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), ethanol precipitated, and then reprecipitated. Samples were run on 6% acrylamide urea gels (60 cm long) with a gradient from 1× to 2× buffer [M. D. Biggin, T. J. Gibson, G. F. Hong, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3963 (1983)]. Gels were fixed and dried before exposing. Binding constants were estimated by cutting out and determining the Čerenkov radiation from sections of a dried gel containing DNA fragments from binding and nonbinding regions of the same lane.
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Epitope Mapping by Chemical Modification of Free and Antibody-Bound Protein Antigen

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A monoclonal antibody bound to a protein antigen slows the rate of chemical modification of amino acid residues located at the epitope. By comparing the degree of acetylation of 18 lysine and 7 threonine residues in free and antibody-bound horse cytochrome c, a discontiguous, conformational epitope was characterized on this protein antigen. The new approach is particularly suitable to probe discontiguous and conformational epitopes, which are difficult to analyze by other procedures.

EPITOPES OF PROTEIN ANTIGENS have been classified as contiguous and discontiguous (*1*, *2*). Contiguous epitopes are composed of residues that are close to each other in the polypeptide sequence, whereas discontiguous epitopes consist of residues that are distant in the polypeptide sequence but adjacent on the protein surface. The term "conformational" designates the dependence of antibody binding on the spatial conformation of the epitope. The classification of epitopes is operational, as it depends on the method used for their detection. In one way or another existing methods are based on the competition for the antibody-combining site between the parent antigen used to mount the immune response and species-related proteins, chemically modified proteins, or peptides (*2*). Here we present a novel approach that complements existing methods for probing epitopes on protein surfaces. The rationale is to compare the relative rate of chemical modification of residues of a protein antigen in the presence or absence of a specific monoclonal antibody and to deduce the location of the epitope from the differential chemical reactivity of amino acid side chains. This type of differential chemical modification was applied successfully to map electron-transfer interaction domains for phys-

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iological redox partners of cytochrome c (*3, 4*). Lysine residues at the binding site for the redox partner were less reactive than lysines at other positions. The same may hold for residues contained in various epitopes of cytochrome c (*2, 5, 6*).

The relative rate of acetylation of lysines and threonines in cytochrome c was measured in the presence or absence of monoclonal antibody 2.61 raised against native horse cytochrome c in SJL mice (*7*). Free and antibody-bound cytochrome c was treated with a trace of [^{3}H]acetic anhydride. On average, only one out of four molecules of cytochrome c was acetylated with ^{3}H in this trace-labeling reaction. By this means, we ruled out the possibility that the acetylation of one group inadvertently affected the rate of acetylation of a neighboring group (*3*). Antibody-binding remained unchanged after trace-labeling. Trace-labeled cytochrome c was fully acetylated with nonradioactive anhydride and mixed with homogeneously ^{14}C-acetylated cytochrome c. The ensuing mixture of chemically homogeneous but isotopically heterogeneous cytochrome c derivatives was subjected to proteolysis, and peptides were separated by high-performance liquid chromatography and sequenced. With this protocol, the degree of acetylation of single residues during trace-labeling could be obtained from ^{3}H/^{14}C ratios (*8, 9*). The degree of acetylation of 18 lysines and 7 threonines was measured (*10*). These residues account for 25% of the sequence and are quite evenly distributed over the entire molecular surface (*11*). Of these residues, only Lys^{60} and Lys^{99} were significantly less reactive in the antigen-antibody complex (Table 1). The decrease of reactivity of Lys^{60} and Lys^{99} was rather small, because free and antibody-bound cytochrome c are in a dynamic equilibrium during acetylation and because the small reagent molecule can probably penetrate between antigen and antibody.

In the crystal the ϵ-amino groups of Lys^{60} and Lys^{99} are brought together to within a distance of 7 Å by folding of the polypeptide chain on the backside of the molecule (*11*). Thus, antibody 2.61 seems to recognize a discontiguous epitope in this region. However, from the acetylation experiment alone we cannot strictly rule out the possibility that residues 60 or 99 or both are outside of the epitope and were made less reactive by a change of conformation induced by the antibody. Therefore, a conventional competition analysis with different cytochromes and CNBr-cleaved fragments was undertaken to clarify this point (Fig. 1 and Table 2). The pattern of cross-reactivities obtained correlates best with sequence changes at Glu^{92}, since all "nonbinders" have a replacement in position 92. Lys^{60} or Thr^{89} contributes little to binding, because the beef protein, which has Lys^{60} and Thr^{89} replaced by Gly, was strongly cross-reacting. However, a derivative of horse cytochrome c with N^{ϵ}-4-carboxy-2,6-dinitrophenyllysine (CDNP-Lys) in position 60 (*12*) showed considerably less cross-reactivity. Steric interference by this bulky substituent with binding to antibody 2.61 strengthens the notion that Lys^{60} is part of the epitope. Changes at

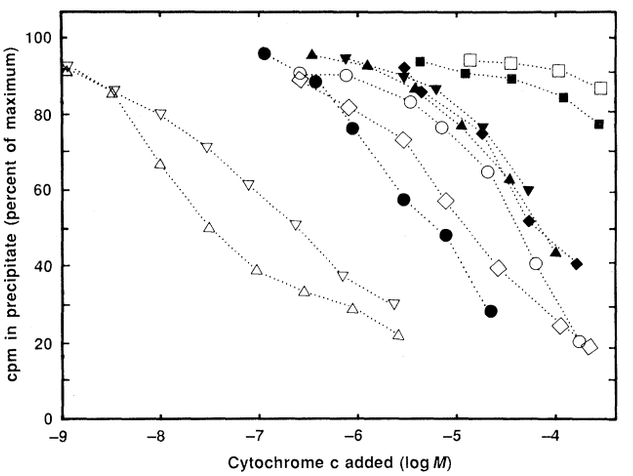

Fig. 1. Competition assay in which various cytochromes c are compared for their ability to inhibit binding of monoclonal antibody 2.61 to ^{125}I-labeled horse cytochrome c. Cytochromes tested were: horse (△), beef (▽), CDNP-Lys^{60} horse (●), rabbit (◇), mouse (○), human (▲), dog (▼), chicken (◆), pigeon (■), and tuna (□). Methods: ^{125}I-labeled horse cytochrome c (24,000 to 35,000 cpm; 2 to 3 ng) was incubated with serial dilutions (1:3) of various unlabeled cytochromes c. Monoclonal antibody 2.61 (50 ng) was added (final volume 300 μl, phosphate-buffered saline buffer, *p*H 7.4, supplemented with 10% normal rabbit serum), and the mixture was incubated at 4°C overnight. Antigen-antibody complexes were precipitated by 50% saturated ammonium sulfate. Precipitate and supernatant were counted in a gamma counter. Concentrations of cytochromes c were determined photometrically.

Table 1. Ratio of the degree of acetylation of lysine and threonine residues of horse cytochrome c free and bound to monoclonal antibody 2.61.

Residue	Ratio*	Residue	Ratio*
Lys^5	0.8	Thr^{58}	1.6
Lys^7	0.6	Lys^{60}	2.6
Lys^8	0.5	Lys^{72}	0.9
Lys^{13}	1.7	Lys^{73}	1.0
Thr^{19}	1.3	Thr^{78}	0.6
Lys^{22}	0.7	Lys^{79}	1.6
Lys^{25}	0.9	Lys^{86}	0.9
Lys^{39}	1.5	Lys^{87}	0.8
Thr^{40}	1.3	Lys^{88}	0.8
Thr^{47}	1.1	Lys^{99}	2.3
Thr^{49}	1.3	Lys^{100}	0.6
Lys^{53}	1.2	Thr^{102}	0.9
Lys^{55}	1.2		

*The average ratio for all residues except Lys^{60} and Lys^{99} is 0.98 ± 0.35. Ratios above 1.7 (two standard deviations above unity) are taken to be significantly different from unity, that is, to indicate lower reactivity in antibody-bound cytochrome c. Values for lysines 22, 55, 60, 72, 73, 86, 87, 88, 99, and 100 were obtained from independent analysis of chymotryptic and thermolytic peptides. The correlation between the two sets of data was $R_{chymotryptic} = 0.81 \times R_{thermolytic} + 0.09$; correlation coefficient = 0.89.

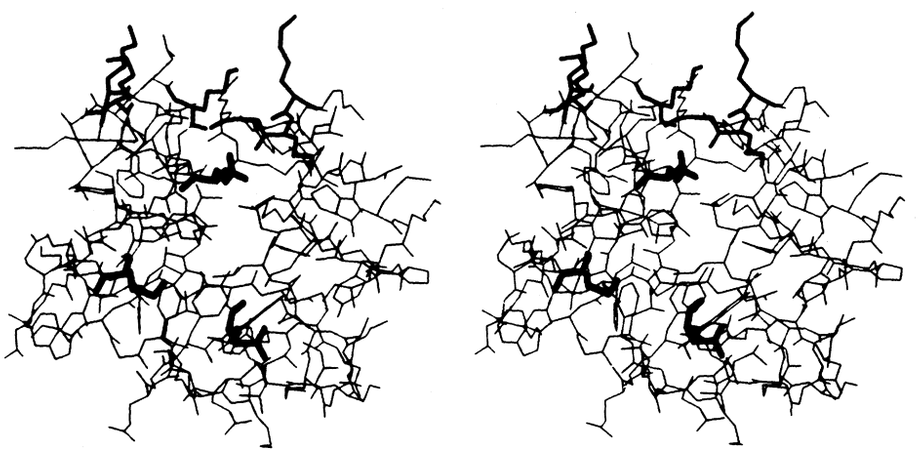

Fig. 2. Stereo view of tuna ferricytochrome c. The three residues marked in very heavy line are Gln^{92} (top), Lys^{99} (left), and Asn^{60} (lower right). They are at positions equivalent to Glu^{92}, Lys^{99}, and Lys^{60} of horse cytochrome c (*11, 18*). The approximate distance between side chains of residues 92 and 99 is 10 Å, between 92 and 60, 14 Å, and between 99 and 60, 7 Å. Residues marked in heavy line are less than 15 Å from Glu^{92} but were equally reactive in free and antibody-bound cytochrome c (top left: Lys^5 and Lys^8; top middle: Lys^{13}; top right: Lys^{87}, Lys^{88}, and Thr^{89}). Atomic coordinates were obtained from Protein Data Bank, Brookhaven National Laboratory. The heme is omitted for clarity.

Table 2. Comparison of amino acid sequences of cytochromes c (*17*). Only residues that differ among the cytochromes listed are shown. The following amino acid abbreviations are used: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; M, methionine; N, asparagine; P, proline; Q, glutamine; S, serine; T, threonine; V, valine; X, CDNP-lysine; Y, tyrosine.

Source	Concentration*	Residue																														
		3	4	9	11	12	15	22	28	44	46	47	50	54	58	60	61	62	83	88	89	92	95	99	100	102	103	104
Horse	1	V	E	I	V	Q	A	K	T	P	F	T	D	N	T	K	E	E	A	K	T	E	I	K	K	T	N	E
Beef	5	—	—	—	—	—	—	—	—	—	—	S	—	—	—	G	—	—	—	—	G	—	—	—	—	—	—	—
CDNP-horse	100	—	—	—	—	—	—	—	—	—	—	—	—	—	—	X	—	—	—	—	—	—	—	—	—	—	—	—
Rabbit	400	—	—	—	—	—	—	—	—	V	—	S	—	—	—	G	—	D	—	—	D	A	—	—	—	—	—	—
Mouse	1,300	—	—	—	—	—	—	—	—	A	—	S	—	—	—	G	—	D	—	—	G	A	—	—	—	—	—	—
Human	2,300	—	—	—	I	M	S	—	—	—	Y	S	A	—	I	G	—	D	V	—	E	A	—	—	—	—	—	—
Dog	3,300	—	—	—	—	—	—	—	—	—	—	S	—	—	—	G	—	—	—	T	G	A	—	—	—	—	K	—
Chicken	3,300	I	—	—	—	—	S	—	—	E	—	S	—	—	—	G	—	D	—	—	S	V	—	—	D	—	S	K
Pigeon	20,000	I	—	—	—	—	S	—	—	E	—	S	—	—	—	G	—	D	—	—	A	A	—	—	Q	—	A	K
Tuna	20,000	—	A	T	—	—	—	N	V	E	Y	S	—	S	V	N	N	D	—	—	G	Q	V	—	S	—	S	

*Estimated relative concentration (horse cytochrome c = 1) that is necessary for 50% inhibition of binding of ^{125}I-labeled horse cytochrome c to antibody 2.61 in the experiment shown in Fig. 1.

Asn^{103} probably do not diminish binding to monoclonal antibody 2.61, as the human and mouse proteins (no change at position 103) behave like the dog and chicken proteins, which have Asn^{103} replaced by Lys and Ser, respectively. Hence, Glu^{92} seems to be the immunodominant residue and possibly an immunogenic residue of horse cytochrome c for SJL mice. Glu^{92} was also the determinant for a class of affinity-purified polyclonal antibodies (*2, 6*). As expected for a discontiguous and conformational epitope, apocytochrome c and the CNBr fragments 66 to 80 and 81 to 104 (*13*) were not competing with the native protein under the conditions of the experiment shown in Fig. 1.

Inspection of a model of horse cytochrome c (Labquip) reveals eight acetylated residues that are less than 15 Å away from Glu^{92}: lysines 5, 8, 13, 60, 87, 88, 99, and Thr^{89} (distances measured along the molecular surface). Given the approximate size of an antibody-combining site that is roughly 700 Å^2 (*14*), any of the above residues might be protected by an antibody directed against Glu^{92}. Quenching of only Lys^{60} and Lys^{99} (*10*) therefore strongly indicates that the epitope for antibody 2.61 extends over the backside (*11*) of the molecule (Fig. 2).

Could a residue of the epitope have escaped detection because its reactivity did not change? A residue at the intermolecular interface must be sterically less accessible for acetic anhydride. Van der Waals' contacts and ionic or hydrogen bonds with residues of the paratope will also decrease reactivity. Preferential binding of acetic anhydride to the intermolecular interface or a large drop of the pK_a of the ε-amino group through binding to the antibody could cancel these effects. Hence, equal reactivity need not indicate necessarily that a residue is outside the epitope. Lower reactivity in the antigen-antibody complex, however, indicates strongly that a surface-located residue is covered by the paratope. Lower reactivity by itself is not a sufficient criterion for direct protection by the antibody.

By a combination of differential chemical modification and classical inhibition experiments an epitope can be outlined in terms of members of two classes of residues: those that are antigenic and/or immunogenic because they are species-specific (Glu^{92}) and those that are covered by the antibody-combining site yet need not be antigenic or immunogenic (Lys^{60} and Lys^{99}). Residues of the latter type far outnumber the former in a conservative protein like cytochrome c. They cannot be detected by a conventional competition assay. Differential modification of surface-located Asp and Glu is also possible (*15*) and may permit more precise mapping of an epitope (*16*).

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7. Monoclonal antibody 2.61 was the product of a hybrid between spleen cells of SJL mice immunized with native horse cytochrome c and the P3U1 myeloma of BALB/c mouse origin. Cells were cultured in Iscove's modified Dulbecco's minimum essential medium supplemented with 10% fetal calf serum; ascites was produced in irradiated (600 rad) DBA/2 mice. Antibody 2.61 was purified by affinity chromatography on a cytochrome c–Sepharose column. The antibody was of the immunoglobulin G1 type, isoelectric point was 8.5, the dissociation constant, $K_d = 0.5 \times 10^{-8} M$ to $1 \times 10^{-8} M$ at 25 °C [determined by radioimmunoassay based on the method of R. S. Farr, *J. Clin. Invest.* **103**, 239 (1958)].
8. The acetylation process was as follows: Oxidized horse cytochrome c (36 nmol) in 1 ml of 80 m*M* KP_i, 70 m*M* NaCl, *p*H 7.8 (experiment A), and cytochrome c (36 nmol) plus monoclonal antibody 2.61 (40 nmol with respect to antibody-combining site) in 1 ml of the same buffer (experiment B) were treated with [^{3}H]acetic anhydride (100 nmol, 694 Ci/mol) at 25°C. Experiments A and B contained in addition 1 μCi of [U-^{14}C]phenylalanine (509 mCi/mol), which also was ^{3}H-acetylated and thereby served as an internal standard nucleophile to monitor slight differences of reaction conditions (*3, 4*). After ^{3}H-labeling, 1 μmol of unlabeled, native horse cytochrome c and 5 μmol of *N*-acetylphenylalanine were added as carriers to facilitate subsequent manipulations. Antibody, cytochrome c, and low molecular weight reaction products were separated by gel filtration (Biogel P-100, 0.39*M* NaP_i, *p*H 2.8, 0.3*M* NaCl). Labeled *N*-acetylphenylalanine was purified by high-performance liquid chromatography [H. R. Bosshard, *J. Mol. Biol.* **153**, 1125 (1981)] and its ^{3}H/^{14}C ratio determined. The ratio in experiment A was 1.6 times that in experiment B. This factor was used to normalize ^{3}H/^{14}C ratios of acetylated lysines and threonines. The degree of acetylation was determined as follows: ^{3}H-labeled cytochrome c plus carrier from experiments A and B (990 nmol total protein in each experiment) were each mixed with uniformly ^{14}C-acetylated cytochrome c [34 nmol, 200 Ci/mol (*4*)]. ^{14}C-Acetylated cytochrome c served as a concentration standard (*3*). Protein mixtures were denatured in 6*M* guanidine-HCl, fully acetylated with excess nonradioactive acetic anhydride (*4*), and treated with hydroxylamine to cleave phenolic *O*-acetyl groups [D. G. Smyth, *J. Biol. Chem.* **242**, 1592 (1967)]. The fully acetylated protein mixtures were digested with thermolysine, V8-protease from *Staphylococcus aureus*, and α-chymotrypsin (*9*), peptides separated by reversed-phase high-performance liquid chromatography (*9*), and sequenced by manual Edman degradation [J. Y. Chang, *Biochem J.* **199**, 557 (1981)]. Radioactive thiohydantoin derivatives were identified by high-performance liquid chromatography and their ^{3}H/^{14}C ratios determined (*9*).
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16. After the present work was completed, we became aware of a very recent publication on epitope mapping in protein antigens by R. Jemmerson and Y. Paterson [*Science* **232**, 1001 (1986)]. These investigators based their analysis of two epitopes of horse cytochrome c on a similar phenomenon, namely, on the different rate of proteolysis of peptide bonds of an epitope in free and antibody-bound cytochrome c. By this approach peptides contributing to the epitope could be revealed. The analysis of Jemmerson and Paterson is limited by the specificity of the protease and by the resistance of the antibody or Fab fragment to proteolysis. The focus of the present procedure is on individual residues of an epitope, the analysis being limited by the distribution of modifiable side chains. Thus, the two procedures complement each other and should, in combination with conventional cross-reactivity measurements, greatly contribute to a more comprehensive analysis of epitopes.
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Leader Peptidase of *Escherichia coli*: Critical Role of a Small Domain in Membrane Assembly

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Leader peptidase spans the *Escherichia coli* plasma membrane with its amino-terminal domain facing the cytoplasm and its carboxyl terminus facing the periplasm. It is made without a cleavable leader sequence. The three apolar domains near the amino terminus of the peptidase are candidates for internal "signal sequences" and they anchor the protein to the lipid bilayer. Oligonucleotide-directed deletion was used to show that only the second domain has an essential function in membrane assembly. While this second apolar domain is crucial for membrane assembly, its continued function when disrupted by arginine suggests that its apolar character per se is not its only important feature.

MANY MEMBRANE PROTEINS ARE synthesized without a cleavable, amino-terminal leader peptide (*1*). Their membrane assembly has been proposed (*2*) to require internal, uncleaved "signal sequences," short amphipathic domains which initiate the translocation of other regions of the protein. Bos *et al.* (*3*) have shown that a viral hemagglutinin contains such a sequence. It has also been suggested (*4*) that membrane proteins may form spontaneous insertion domains which integrate into the bilayer. The sequence and structural characteristics of such domains have yet to be determined.

Like many proteins of the inner membrane of *Escherichia coli*, leader peptidase has no cleaved leader peptide (*5*). This enzyme spans the plasma membrane, with a short domain near the amino terminus exposed to the cytoplasm and a large, polar carboxyl-terminal domain exposed to the periplasm (*5*). The active site of the enzyme is at the periplasmic membrane surface (*6*). However, its amino-terminal domain, which faces the cytoplasm, is essential for its in vitro catalytic activity (*7*). We have used drugs and mutants to study its membrane assembly in vivo. Leader peptidase requires both the electrochemical membrane potential (*8*) and the function of the *sec*A and *sec*Y genes (*9*) for membrane assembly. As with essentially all exported bacterial proteins, its membrane insertion is not coupled to ongoing polypeptide chain growth (*8*). Prior to membrane assembly, leader peptidase is in a conformation which is more readily degraded by protease than the final, transmembrane form (*8*). Deletion of 182 amino acids from the carboxyl-terminal end of the protein (*10*) blocks its membrane assembly.

Our working model of the orientation of leader peptidase across the plasma membrane (Fig. 1A) is based on the sequence of the protein (*5*) and on its topology (*11*). Early studies (*5*) revealed that trypsin can only remove approximately 60 residues from the amino terminus of leader peptidase in sealed, inverted plasma membrane vesicles. More recently (*11*), it was shown that all but the amino-terminal part of leader peptidase, including the first two apolar domains, is accessible to digestion by proteinases added to intact spheroplasts. To test which apolar domains of leader peptidase form an internal, uncleaved "signal," which are essential for stable anchoring to the membrane, and which affect catalytic activity, we have inserted arginyl residues into these regions, or deleted them entirely, by oligonucleotide-directed mutagenesis (Fig. 1B). These mutant leader peptidase genes are called "XR," where X is the amino acid residue which is replaced by arginine, or ΔX-Y, indicating deletion of residues X to Y. Of the ionizable amino acids, arginine was chosen because its pK_a (dissociation constant) is the furthest from neutrality. Each mutant leader peptidase gene was sequenced in M13mp8 and transferred into a plasmid where its expression was regulated by the *ara* promoter. The plasmids bearing these mutants under arabinose promoter regulation are termed pRDXR or pRDΔX-Y.

The membrane assembly of each mutant leader peptidase was assayed in intact cells. Addition of arabinose to *E. coli* pRD9R/HJM114 induces the synthesis of leader peptidase 9R. Synthesis was assayed by labeling cells with [^{35}S]methionine, immunoprecipitation with antiserum to leader peptidase, and SDS-PAGE (polyacrylamide gel electrophoresis) and fluorography (Fig. 2B, first lane). To determine whether leader peptidase 9R can assemble across the plasma membrane, labeled cells were treated with tris, sucrose, and EDTA to permeabilize the outer membrane, then incubated with trypsin. Leader peptidase was accessible to trypsin digestion (Fig. 2B), indicating that it had assembled across the plasma membrane. Most cell proteins (Fig. 2A) and, in particular, an abundant cytoplasmic marker protein (the amino-terminal fragment of ribulokinase) (Fig. 2B) remained inaccessible to digestion unless the inner membrane permeability barrier was abolished by ultrasound. This confirmed that the plasma membrane remained intact during these studies.

Three further criteria were used to ascertain that leader peptidase 9R assembles normally into the plasma membrane: (i) Disruption of the most apolar segment of the protein might have led to its complete export into the periplasm. To test this, cells (Fig. 3A, lane 4; total protein) were treated with tris, sucrose, and EDTA to permeabilize their outer membrane and release the periplasmic contents (Fig. 3A, lane 6). Leader peptidase 9R was entirely recovered with the treated cells (Fig. 3B, lane 5) and not with the periplasm (Fig. 3B, lane 6). (ii) Trypsin treatment of right-side-out inner membrane vesicles from such cells not only degraded the leader peptidase 9R (Fig. 4,

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