Isolation of a Bacterial Membrane Protein, Nectin, Essential for the Attachment of Adenosine Triphosphatase*

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CARL BARON AND ADOLPH ABRAMS
From the Department of Biochemistry, University of Colorado School of Medicine, Denver, Colorado 80220

SUMMARY

We have isolated from Streptococcus faecalis plasma membranes a new protein, termed nectin, which is required for the attachment of the membrane enzyme, ATPase. Both nectin and membrane-bound ATPase are released from the native membranes by a procedure, described previously, which depends on repeated washing in dilute buffer without Mg++. Resolution of the solubilized nectin and ATPase can then be accomplished by gel filtration through agarose. The ATPase, separated from nectin by passage through agarose, fails to reattach to enzyme-depleted membranes unless nectin is added. The amount of ATPase that recombines with the depleted membranes is proportional to the amount of nectin added until the membrane receptor sites for the enzyme become nearly saturated. Nectin is heat labile and has an ultraviolet absorption spectrum typical of a protein. By the method of gel filtration through Sephadex, we estimate that its molecular weight is 37,000.

Except for a few enzymes little is known of the nature or specific function of proteins in plasma membranes. Most membrane proteins are probably not enzymes but function instead in ways that determine the specific physical properties and organizational features of membranes, or act as specific transport carriers. The work presented here deals with the identification and isolation of a membrane protein whose function is organizational rather than catalytic.

In earlier studies we showed that the ATPase associated with the plasma membrane of Streptococcus faecalis could be released from the membrane and then fully reattached (1–3). This membrane enzyme, which has since been purified to homogeneity and fairly well characterized (4–6), is believed to function in the active transport of monovalent cations (7). We wish now to report the isolation of a protein from the S. faecalis membrane which is required for the reattachment to the membrane of the solubilized ATPase. We have named this new protein nectin1 both for convenience and to signify what we consider to be its specific function of proteins in plasma membranes. Most membrane proteins are probably not enzymes but function instead in ways that determine the specific physical properties and organizational features of membranes, or act as specific transport carriers. The work presented here deals with the identification and isolation of a membrane protein whose function is organizational rather than catalytic.

To isolate nectin it must first be released in soluble form from the S. faecalis plasma membranes. This is accomplished by the same procedure we have used previously to liberate the membrane-bound ATPase (2). Cells harvested from the stationary phase of growth (7 g wet weight) were converted to protoplasts by treatment with lysozyme in 0.4 M glycylglycine, pH 7.2. The protoplasts were then lysed by osmotic shock in cold 2 M LiCl and then with 1 M Tris-Cl, pH 7.5, without Mg++. This washing procedure leads eventually to an abrupt release of the membrane-bound ATPase together with some other membrane proteins into about the sixth wash fluid, leaving a membrane residue almost completely depleted of the enzyme (2). The solubilized membrane proteins thus obtained were then precipitated with ammonium sulfate between 35 and 80% saturation at 4°C, redissolved in a small volume of 33 mM Tris-Cl, pH 7.5, and finally dialyzed against the same buffer. This preparation, which contains sATPase2 and a number of other solubilized membrane proteins, including nectin, is referred to as crude sATPase. As shown in Fig. 1, and also in earlier studies (3), when concentrated crude sATPase is added to the enzyme-depleted membranes in the presence of Mg++, the enzyme reattaches to the membrane. Chromatography on agarose in the absence of Mg++, as described in Fig. 2, resolves the crude sATPase into a peak containing sATPase, hereinafter called agarose-purified sATPase, and a peak containing nectin.3 The agarose-purified sATPase, now separated from nectin, fails almost completely to reattach to the enzyme-depleted membranes even in the presence of Mg++ (Fig. 1). However, by adding back the fractions containing nectin, the ability of the agarose-purified sATPase to reattach to the membrane is restored (Figs. 2 and 3). This effect of nectin is the basis for its detection and assay.

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1 The word nectin is taken from the Latin, nectere, which means to bind and is the origin of the word "connect."

2 The abbreviation used is: sATPase, solubilized ATPase.

3 Early in these studies we found that if 0.01 M Mg++ was present in the eluting buffer, then nectin and sATPase do not resolve on agarose columns.
FIG. 2. Resolution of sATPase and nectin by chromatography on agarose. Crude sATPase (100 units) was layered on a column of agarose (Bio-Gel A, 1.5 m, 100 to 200 mesh; Bio-Rad, Richmond, California), 2.5 X 83 cm, and was eluted with 33 mm Tris-Cl, pH 7.5. Each fraction contained 4.9 ml. ATPase activity, in the column fractions, was assayed as previously described (7). The units of ATPase are given per column fraction. This agarose-purified sATPase was then concentrated to about 2 ml with the Collodion Bag Apparatus (Carl Schleicher and Schuell Company, Keene, New Hampshire). The amount of nectin is given in terms of the units of ATPase that reattach when 0.2 ml of a column fraction is added to a mixture of agarose-purified sATPase and depleted membranes. In the assay of nectin, depleted membranes (0.2 mg), agarose-purified sATPase (0.4 units), and 0.2 ml of a column fraction were incubated for 15 min at 38° in a final volume of 0.3 ml. This solution contained 0.01 M Mg++ and 0.1 M Tris-Cl, pH 7.5. The amount of ATPase that became attached was then determined by centrifugation as previously described (3). In the measurements of nectin a small blank has been subtracted which represents the amount of ATPase which reattached to membranes in the absence of added nectin.

Fig. 3 shows that the amount of agarose-purified sATPase that reattaches to a given amount of depleted membranes is proportional to the level of nectin added until the depleted membranes become fully saturated with ATPase. The specific enzymatic activity of the fully reconstituted ATPase-membrane complex (1.67 units per mg of protein) obtained at high levels of nectin was the same as that of the original native enzyme-membrane complex (1.70 units per mg of protein). This indicates that nectin restores ATPase to receptor sites on the membrane specific for the enzyme. The nectin-dependent formation of the ATPase-membrane complex also restores the sensitivity of the enzyme to inhibition by dicyclohexylcarbodiimide (7). It has been shown previously that this compound inhibits only the membrane-bound form of the enzyme. Thus it appears that the functional relationships existing in the native enzyme-membrane complex are re-established by nectin.

We have observed that reconstitution of the ATPase-membrane complex brought about by adding nectin to a mixture of agarose-purified sATPase and depleted membranes, in the presence of Mg++, takes place readily at 38° but fails at 0°. However, when crude sATPase (which already contains nectin) is used for reconstitution, attachment of ATPase to the membrane is equally efficient at 0 and 38°. We interpret this finding to mean that a temperature-sensitive interaction between agarose-purified sATPase and added nectin must take place prior to formation of the ATPase magnesium nectin membrane complex.

Nectin is heat-labile. It is inactivated 90% by heating for 5 min at 90° and 60% inactivated by heating for 10 min at 50°. The ultraviolet spectrum of partially purified nectin has an absorption maximum at 272 mp and a minimum at 246 mp. It is not sensitive to RNAse. All of the above properties suggest

Fig. 4. Molecular weight estimate of nectin on Sephadex G-100. One milliliter of a concentrated nectin solution (Fig. 3) was layered on a column of Sephadex G-100 (particle size: 40 to 120 μ; Pharmacia, Uppsala, Sweden), 1.5 X 88 cm, and was eluted with 33 mm Tris-Cl, pH 7.5, in fractions of 2.2 ml. The nectin-containing fractions were determined as described in Fig. 2. The dotted lines below the abscissa indicate the positions of the protein standards: bovine serum albumin (67,000), ovalbumin (47,500), and myoglobin (17,800). Nectin is eluted in a volume corresponding to a molecular weight of 37,000 as indicated in the inset.
that nectin is a protein. We have estimated that the molecular weight of nectin is about 37,000. This estimation is based on gel filtration through Sephadex G-100 using suitable protein markers (Fig. 4) (9). Work is now in progress to obtain nectin in pure form and to examine the nature of its interaction with the ATPase and its possible role in active transport. It is worth noting that the self assembly in vitro of two purified membrane proteins, ATPase and nectin, and depleted membrane that we have described here may be similar to processes occurring during membrane biosynthesis in vivo.

REFERENCES


Cross-Reactions of Adenosine 3',5'-Monophosphate-dependent Protein Kinase Systems from Rat Liver and Rabbit Skeletal Muscle*

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Hiroshi Yamamura, Akira Kikusui, and Yasutomi Nishizuka

From the Department of Biochemistry, Kobe University School of Medicine, Kobe, Japan

SUMMARY

Protein kinases and regulatory proteins (R-proteins) which are capable of binding adenosine 3',5'-monophosphate (cyclic AMP) are partially purified from rat liver and rabbit skeletal muscle soluble fractions. The activity of either one of these kinases is almost totally depressed by R-protein from the homologous as well as from the heterologous tissue. As described earlier, cyclic AMP activates the inactive kinase by binding to R-protein resulting in the release of active kinase. These protein kinases phosphorylate the same specific serine and threonine residues of histone. Salmon sperm protamine and rabbit skeletal muscle glycogen phosphorylase b kinase serve as phosphate acceptors for both kinases. A possible role of the protein kinase system in controlling cellular activities is also discussed briefly.

Walsh, Perkins, and Krebs (1) have described that adenosine 3',5'-monophosphate greatly stimulates glycogen phosphorylase b kinase (protein kinase) obtained from rabbit skeletal muscle. The occurrence of cyclic AMP-dependent protein kinase which phosphorylates preferentially histone and protamine has been shown subsequently in rat liver (2), bovine brain (3), rat adipose tissue (4), and various other vertebrate and invertebrate tissues (5), as well as in bacteria (6). The preceding reports from this laboratory (7, 8) have shown that the activity of protein kinase obtained from rat liver is controlled by reversible association with a regulatory protein which is capable of binding cyclic AMP. The attachment of R protein to protein kinase almost totally dephosphorylates its activity and converts the kinase to an inactive R-protein-kinase complex. Cyclic AMP activates such inactive kinase by binding to the R-protein molecule resulting in the release of active kinase from R-protein. Recently, a similar mechanism of action of cyclic AMP has been proposed also with bovine adrenal protein kinase by Gill and Garren (9), with rabbit skeletal muscle protein kinase by Krebs (10), and also with rabbit reticulocyte protein kinase by Tao, Salas, and Ljuman (11). Further studies in this laboratory on cyclic AMP-dependent protein kinases from various mammalian tissues have revealed that this mechanism of action of cyclic AMP operates in most tissues and organs, and that protein kinases and R-proteins are homologous, as well as from heterologous proteins.

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1 The abbreviations used are: R-proteins, regulatory proteins; cyclic AMP, adenosine 3',5'-monophosphate.

*The preparation of glycogen phosphorylase b employed for this experiment contained a trace amount of 3'-AMP, and produced 736 cpm of glucose 1-phosphate in the absence of activating system under these conditions. This value was subtracted from the corresponding experimental values.
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Carl Baron and Adolph Abrams


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