Microsomal Ouabain-Binding Factor in Electroplax Extracts[1] (36587)

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Active sodium and potassium ion transport is believed to depend on the enzyme complex, $(Na^+ + K^+)$-adenosinetriphosphatase (1). One of the most important correlations linking the sodium pump to this enzyme is the fact that ouabain and other cardioactive steroids inhibit the transport function in whole cells and the $(Na^+ + K^+)$-ATPase activity in whole cells and in microsomal preparations (2). In addition, ouabain binds to microsomal $(Na^+ + K^+)$-ATPase in a number of tissues (3). Binding of ouabain to electroplax microsomes enriched in enzyme activity has been correlated with inhibition of catalytic activity (4) and alteration of other properties of the enzyme (5). Although there is some controversy regarding the precise mechanism of binding, it is clear that the rate of ouabain binding is dependent on interactions of the enzyme complex with substrates, products and ligands (6–9). Therefore, it is believed that the ouabain-binding factor (OBF) is both a part of the enzyme complex and of the sodium pump molecular machinery.

However the structural relationship of the OBF within the enzyme–pump complex is not known. An understanding of the catalytic properties and structure of the enzyme and of the way in which ATP hydrolysis is linked to the transport function depends on separation and purification of the various components. For this purpose, the use of enzyme catalytic activity alone as a marker is insufficient because other linking components not necessary for catalysis may be lost and because often there are large losses in catalytic activity during separation procedures. Separation of OBF from red cell membranes has been reported (10). The present work is an attempt to solubilize and separate bound ouabain from electroplax microsomes under conditions that preserve the ouabain in bound form.

Methods. Microsomal preparations of $(Na^+ + K^+)$-ATPase were obtained from *Electrophorus electricus* electroplax as described (11). Initial rates of ATPase activity were measured at 23° by a spectrophotometric method based on oxidation of NADH (4). Protein measurements in stock suspensions were performed according to an established method (12). Chromatography was performed with Sepharose 4B[2] equilibrated with the eluent, 5 m*M* Tris–HCl (pH 7.2) at 2°. Column dimensions were 27.5 × 1 cm. Elution was carried out at 2° by downward flow at 1 ml/20 min and 1 ml fractions were collected. Void volume was 7 or 8 ml in different experiments as indicated by the elution of blue dextran.[2] A portion of each fraction was used to measure the protein content by the method for dilute protein samples as described (12). Bovine serum albumin dissolved in the eluent was used as standard. It was determined that Lubrol WX[3] 0.25% (w/v), did not interfere with measurement of standard protein samples as low as 2.5 μg/ml. Another portion of each fraction was transferred to naphthalene dioxane scintillation counting solution for radioactivity measurements in a Packard Tricarb spectrome-

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[2] Obtained from Pharmacia Fine Chemicals, Inc., Piscataway, NJ.

[3] Obtained from ICI America, Inc., Stamford, CT.

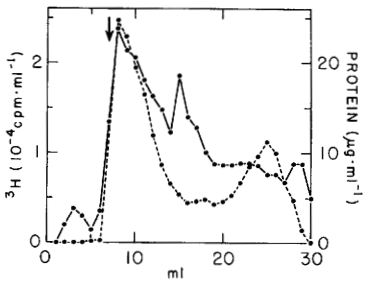

FIG. 1. Elution pattern of bound ouabain (P_i-dependent). Ouabain-microsomes were obtained as described in the text. The incubation medium for ouabain binding contained in addition, 3 m*M* Tris–PO_4 and 3 m*M* $MgCl_2$. Ouabain-microsomes, containing 0.65 nmoles bound ^3H/mg protein, were incubated in 0.25% (w/v) Lubrol WX (1:1 vs protein), 5 m*M* Tris–HCl (pH 7.2), and 0.1 m*M* 2.3-dimercaptopropanol in a volume of 0.4 ml for 30 min at 2°. The mixture was centrifuged at 50,000*g* for 30 min at 2°. The supernatant (see Table I, expt. A) was applied to Sepharose 4B for chromatography as described in the text. The arrow indicates the elution of dextran blue. (●- -) radioactivity; (●—) protein.

ter. No quenching was observed by the method of external standardization. In other experiments, a half of each fraction was used to assay ATPase activity.

Batches of microsomes were labeled with ouabain as described (4) but with certain modifications. Microsomes, 4.55 mg protein, were incubated with 0.1 m*M* [^{3}H]-ouabain (sp act 6.22 × 10^2 Ci/mole) in 75 m*M* Tris–HCl (pH 7.4) plus other additions as indicated in a volume of 1 ml for 60 min at 23°. The mixture was centrifuged for 60 min at 50,000*g* and 2°. The pellet was washed three times by resuspension in 30 ml of 5 m*M* Tris–HCl (pH 7.2), 1 m*M* $MgCl_2$ and resedimented. The washed pellet was finally suspended in 5 m*M* Tris-HCl and stored overnight in liquid nitrogen. Prior to use, the ouabain-microsomes were again sedimented and the supernatant, which contined no significant radioactivity, was decanted.

Results and Discussion. Figure 1 shows the elution pattern of bound ouabain which has been extracted into Lubrol. In this experiment, ^{3}H-ouabain is first bound to microsomes in the presence of Mg^{2+} and P_i. The ouabain-microsomes are then incubated in 0.25% Lubrol (w/v) (1:1 vs protein) for 30 min at 2°. After sedimentation at 50,000*g* for 30 min at 2°, 84% of the radioactivity and 33% of the protein are found in the supernatant fraction (Table I). It was determined that free ouabain applied to the Sepharose 4B column is eluted almost entirely in fractions 17 to 30. About 0.8% is eluted in fractions 14 to 20 and less than 0.005% in fractions 7 to 14. When the Lubrol extract is chromatographed, 54% of the applied radioactivity is found in fractions 7 to 14 (zone I) along with 48% of the applied protein. All of the ouabain in this zone is presumed to be bound to microsomal components. An additional 15% of the applied radioactivity is found in fractions 14 to 30 (zone II). It is likely that almost all the ouabain in zone II is also bound to particles or aggregates of varying sizes. It is unlikely that any significant portion of the radioactivity in fractions 17–30 (zone III) is bound since we have found that after passage of the pooled fractions of zone III through Sephadex G-10, no radioactivity is present in the exclusion volume with the protein.

We do not yet know what proportions of bound ouabain dissociate during Lubrol extraction prior to chromatography and during chromatography. Therefore, we cannot determine what proportion of the free ouabain in zone III derive originally from the microsomes sedimented during the Lubrol extraction. This information will be necessary to determine the total amount of OBF that is extracted into the Lubrol supernatant fraction. However, on the basis of available information, we can conclude that at least 53% (zones I and II) of the orginal microsomal bound ouabain is extracted into Lubrol and preserved in bound form after chromatography. At least 45% (zone I) of the original bound radioactivity is found in fractions containing only 16% of the original protein, representing a purification of at least 2.8-fold in this pool.

Extracts of ouabain-microsomes in which the ouabain is first bound by the substrate-dependent path exhibit similar distributions of protein and radioactivity (Fig. 2). However, the total recovery of bound ouabain after

TABLE I. Extraction of Microsomes with Lubrol and Separation on Sepharose 4B Column.[a]

Expt.	Total	% Pellet	% Super	Applied to column	% I[b]	% II[b]	% III[b]
A.							
Bound ^3H	0.64 nmoles	16	84	0.40 nmoles	54	15	33
Protein	0.99 mg	67	33	0.30 mg	48	30	38
B.							
Bound ^3H	0.46 nmoles	26	74	0.25 nmoles	37	12	46
Protein	1.02 mg	59	41	0.30 mg	41	28	31
C.							
$(Mg^{2+}+Na^+ + K^+)$ ATPase	960 U[c,d]		47	72 U[c,e]	7.3	0	0
(Mg^{2+})—ATPase	58 U[c,d]		62	21 U[c,e]	23	0	0
Protein	1.02 mg		56	0.42 mg	50	33	32

[a] Microsomes were treated with 2.5% Lubrol WX (1:1 vs protein). The conditions for experiment A are described in Fig. 1 and those for experiment B in Fig. 2. In experiment C, native microsomes were treated with Lubrol and the supernatant was chromatographed as in Fig. 1. The left part of the Table shows total quantities in the complete Lubrol suspension of microsomes and the percentages found in the pellet and supernatant portions after centrifugation. The right part shows total quantities applied to the column and the proportions (relative to quantities applied) eluted in different zones.

[b] Zone I consists of column fractions 7–14, zone II, 14–20, zone III, 17–30. Free ouabain is eluted in zone III when applied separately.

[c] U = 1 nmole ATP hydrolyzed per minute at 23°.

[d] Values are for total suspension 30 min after addition of 0.25% Lubrol and prior to sedimentation. The Lubrol concentration in the assay medium is 0.005%. Corresponding values for native microsomes are: $(Mg^{2+} + Na^+ + K^+)$, 2373 U; (Mg^{2+}), 160 U.

[e] Values are for activity of Lubrol extract stored at 2° for 24 hr and assayed with the eluted fractions. Corresponding values for the extract assayed immediately as applied to column are: $(Mg^{2+} + Na^+ + K^+)$, 338 U; (Mg^{2+}), 27 U.

chromatography is smaller. Zones I and II contain 35% of the original bound radioactivity while 27% of the original bound ouabain is found in zone I together with 17% of the original protein. These data suggest that the ouabain bound in the P_i-dependent path (Fig. 1) is more stable than that bound in the substrate-dependent path. It is of interest that the recoverable bound ouabain exhibits identical elution patterns despite the different initial binding conditions.

It is important to determine in what way the functional activities of the pump–enzyme complex are altered or inactivated by the extraction and separation procedures employed. A Lubrol extract was incubated with ^3H-ouabain, Mg^{2+} and P_i and chromatographed on Sepharose 4B (Fig. 3). The elution pattern of radioactivity is similar to that in Figs. 1 and 2. In this experiment a large excess of ouabain was used accounting for the large peak of free ouabain in zone III. In the fractions of peak binding, Nos. 7 and 8, the average is 0.57 nmoles ouabain/mg protein, which represents a lower limit for the binding capacity of the extract. This minimum value is at least the specific binding capacity of the native microsomes (0.65 nmoles/mg protein, Table I, Expt. A).

The effects of Lubrol on enzyme activity were studied. Addition of 0.25% Lubrol to native microsomes results in about 60% inhibition of both the Mg^{2+}-dependent and $(Na^+ + K^+)$-ATPase activities within 30 min at 2° (Table I, Expt. C). About 47% of the residual total activity is found in the

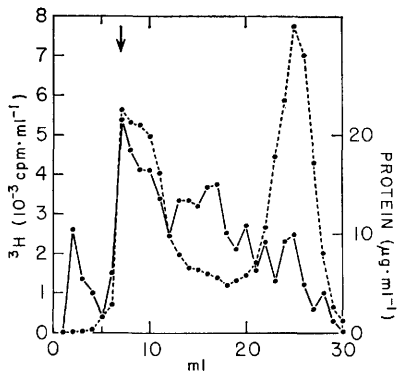

FIG. 2. Elution pattern of bound ouabain (substrate-dependent). Ouabain-microsomes were obtained as described in the text. The incubation medium for ouabain binding included, in addition, 2.5 m*M* Tris–ATP, 2.5 m*M* $MgCl_2$, and 75 m*M* NaCl. Ouabain-microsomes, containing 0.44 nmoles bound ^{3}H/mg protein, were incubated in Lubrol and the extract (see Table I, Expt. B) was chromatographed on Sepharose 4B as in Fig. 1. The arrow indicates the elution of dextran blue. (●- -) radioactivity; (●—) protein.

Lubrol supernatant fraction, representing about 28% of the total activity in the original microsomes. After 24 hr storage at 2°, the Lubrol extract contains about 3% of the original total activity and, of this, about one-third is Mg^{2+}-dependent.

In addition, the stored Lubrol extract loses its ability to bind ouabain. In an experiment performed as in Fig. 3 but using Lubrol extract stored for 24 hr at 2°, no significant quantity of bound ouabain was recovered after chromatography.

A Lubrol supernatant extract of native microsomes was also applied immediately to Sepharose 4B for chromatography (Table I, Expt. C, fig. not shown). Although only 0.2% of the total activity in the original microsomes was recovered, all of this activity was found only in fractions 8 to 11 which correspond to the peak fractions for bound ouabain. The highest specific activity found for total ATPase was 55.5 nmoles/mg/min in fraction 8, which is 2.4% of the starting specific activity (2.34 μmoles/mg/min, Table I). Mg^{2+}-dependent ATPase was also found only in the same fractions and the average in each was about one-half of the total activity. These values are too low to permit adequate distinction from the Na^+-dependent enzyme. Thus, in contrast to recovery of bound ouabain, recovery of enzyme activity is very poor under these conditions. But it is significant that the portion which is recoverable exhibits the same elution pattern as does bound ouabain.

These data show that: (1) ouabain binds to Lubrol "solubilized" components; (2) the ouabain bound to components after Lubrol extraction has the same elution pattern as that bound to microsomes prior to extraction; and (3) the preservation of bound ouabain is much greater than that of catalytic activity under these conditions. These facts mean that under certain conditions which inactivate the enzyme, the isotopically labeled OBF may be an advantageous marker for purification of the pump–enzyme complex and possibly for separation of components for further studies.

Summary. Lubrol WX extracts microsomal-bound ouabain-[^{3}H] into the 50,000*g* supernatant fraction. The bound ouabain can be separated by chromatography with Sepharose and recovered in parallel fractions

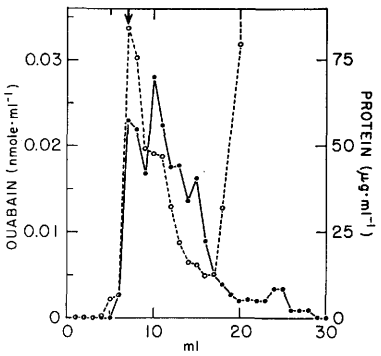

FIG. 3. Ouabain-binding to Lubrol extract of microsomes. Microsomes, 2.047 mg protein, were incubated with 2.047 mg Lubrol WX in 5 m*M* Tris–HCl (pH 7.2) in a volume of 1 ml for 30 min at 2°. The mixture was centrifuged for 30 min at 50,000*g* at 2°. The supernatant was removed and incubated in 2.5 m*M* Tris–PO_4, 2.5 m*M* $MgCl_2$ and 0.1 m*M* [^{3}H]-ouabain (sp act 2.5 × 10^2 Ci/mole) for 45 min at 26°. A 0.4 ml portion, representing half of the ouabain-treated extract, and containing 0.544 mg protein and 0.1 m*M* added 2,3-dimercaptopropanol was applied to Sepharose 4B for chromatography as in Fig. 1. (○- -) ouabain; (●—) protein.

with recoverable adenosinetriphosphatase activity. Ouabain also binds to supernatant components in the Lubrol extracts and this bound ouabain has the same elution pattern as that bound to native microsomes prior to extraction with Lubrol. The proportion of bound ouabain that is recovered after chromatography is much greater than that of enzyme activity. Under these conditions, bound ouabain is a useful marker for enzyme extraction despite inactivation of the enzyme.

1. Skou, J. C., Biochim. Biophys. Acta **23**, 394 (1957).

2. Glynn, I. M., Pharmacol. Rev. **16**, 381 (1964).

3. Whittam, R., and Wheeler, K. P., Annu. Rev. Physiol. **32**, 21 (1970).

4. Albers, R. W., Koval, G. J., and Siegel, G. J., Mol. Pharmacol. **4**, 324 (1968).

5. Siegel, G. J., Koval, G. J., and Albers, R. W., J. Biol. Chem. **244**, 3264 (1969).

6. Siegel, G. J., and Josephson, L., Eur. J. Biochem. **25**, 323 (1972).

7. Hansen, O., Jensen, J., and Norby, J. G., Nature (London) New Biol. **234**, 122 (1971).

8. Lindenmayer, G. E., and Schwartz, A., Arch. Biochem. Biophys. **140**, 171 (1970).

9. Sen, A. K., Tobin, T., and Post, R. L., J. Biol. Chem. **244**, 6596 (1969).

10. Dunham, P. B., and Hoffman, J. F., Proc. Nat. Acad. Sci. U.S.A. **66**, 936 (1970).

11. Albers, R. W., Fahn, S., and Koval, G. J., Proc. Nat. Acad. Sci. U.S.A. **50**, 474 (1963).

12. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

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