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OUABAIN-BINDING AND PHOSPHORYLATION OF (Na^++K^+)ATPase TREATED WITH *N*-ETHYLMALEIMIDE OR OLIGOMYCIN

CSABA HEGYVARY

Department of Physiology, Rush Medical College, Chicago, Ill 60612 (U S A)

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Summary

Ouabain-binding and phosphorylation of (Na^+ + K^+)-ATPase (EC 3 6 1 3) of the plasma membranes from kidney were investigated after treatment with *N*-ethylmaleimide or oligomycin Either of these inhibitors brought about the following changes the phosphoenzyme, formed in the presence of Na^+, Mg^{2+} and ATP became essentially insensitive to splitting by K^+ but was split by ADP One mole of this ADP-sensitive phosphoenzyme bound one mole of ouabain but the enzyme-ouabain complex was less stable than in the native enzyme primarily because the rate of its dissociation increased Ouabain was bound to the ADP-sensitive phosphoenzyme in the presence of Mg^{2+} alone and addition of inorganic phosphate enhanced both the rate of formation and the steady-state level of the enzyme-ouabain complex The inhibitors did not affect the properties of this second type of complex

Both in the native enzyme and in the enzyme treated with the two inhibitors inorganic phosphate enhanced ouabain binding by phosphorylating the active center of the enzyme as shown (a) by mapping the labeled peptides from the enzyme after peptic digestion, (b) by inhibition of this phosphorylation with Na^+ and (c) by the 1 1 stoichiometric relation between this phosphorylation and the amount of bound ouabain

Unlike the phosphoenzyme, the binding of ouabain remained sensitive to K^+ in the enzyme treated with the inhibitors K^+ slowed ouabain-binding either in the presence of Na^+, Mg^{2+} and ATP or of Mg^{2+} and inorganic phosphate A higher concentration of K^+ was needed to slow ouabain-binding than to stimulate dephosphorylation This finding is interpreted as being an indication of separate sites for K^+ on the enzyme a site(s) with high K^+-affinity which

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Abbreviations (Na^+ + K^+)-ATPase, (Na^+ + K^+)-activated ATPase (ATP phosphohydrolase, EC 3 6 1 3), E_1-P, phosphorylated (Na^+ + K^+)-ATPase sensitive to splitting by ADP, E_2-P, phosphorylated (Na^+ + K^+)-ATPase sensitive to splitting by K^+, CDTA, (1,2-cyclohexylenedinitrilo) tetraacetate, EGTA, ethylene glycol *bis* (β-aminoethyl)-*N,N* tetraacetate

stimulates dephosphorylation, another site(s) with moderate K^+-affinity which inhibits ouabain-binding Inhibitors may enhance formation of the ADP-sensitive phosphoenzyme by blocking interaction between K^+ and the site(s) with high affinity

Introduction

($Na^+ + K^+$)-ATPase (EC 3 6 1 3) is an enzymatic component of the Na^+ pump that probably converts the chemical energy of ATP into osmotic work [1,2] The hydrolysis of ATP proceeds through a cycle of phosphorylation and dephosphorylation of an aspartyl residue at the active center [3—6] Phosphorylation requires Na^+ and Mg^{2+}, dephosphorylation is stimulated by K^+ in the native enzyme The phosphoenzyme appears to have at least two major conformations, though several other reactive states were postulated recently [7] The first conformation, (E_1-P), can react with ADP to resynthesize ATP and it is not split by K^+ The second conformation, (E_2-P), is split by K^+ and can equilibrate with inorganic phosphate [7] In the native enzyme primarily, if not exclusively, E_2-P is formed, but E_1-P accumulates if the enzyme is poisoned with *N*-ethylmaleimide or oligomycin [8,9] The relation between these two forms is obscure E_1-P may be a precursor of E_2-P [8,9], or it may represent an alternative pathway [10] for splitting ATP The native enzyme can be phosphorylated by P_i also, this reaction is inhibited by Na^+ and enhanced by K^+ Cardioactive glycosides, such as ouabain, specifically inhibit the enzyme by binding to and stabilizing the phosphoenzyme, formed either from ATP or from P_i [11—13] Some ouabain can be bound apparently without phosphorylation in the presence of Mg^{2+} alone [12] though catalysis of this process by trace amounts of P_i adsorbed to the membranes has never been excluded It is convenient and customary to distinguish enzyme-ouabain complexes by the ligands that were present at binding Thus type I complex is formed in the presence of Na^+, Mg^{2+} and ATP, and type II complex in the presence of Mg^{2+} and P_i [14] This operational definition will be used in this paper also For the native enzyme the formation of both type I and type II complex means binding of ouabain to E_2-P In most previous reports the binding of ouabain to the native enzyme was investigated, though it was recognized that *N*-ethylmaleimide-treated enzyme can also bind ouabain [15—17]

This paper describes some characteristics of ouabain-binding to ($Na^+ + K^+$)-ATPase poisoned with *N*-ethylmaleimide or oligomycin Membranes treated with these inhibitors bound some ouabain in the presence of Mg^{2+} alone and the rate and the steady-state level of ouabain-binding rose if Na^+ and ATP or P_i were also added together with Mg^{2+} Kinetic data indicated that the (E_1-P)-ouabain complex was less stable than the (E_2-P)-ouabain complex with regard to the dissociation of ouabain Since K^+ slowed the rate of ouabain-binding to E_1-P without actually splitting this phosphoenzyme, there may be two types of K^+ sites one for enhancing dephosphorylation, another for inhibiting ouabain-binding Finally it was shown directly that the *N*-ethylmaleimide treated enzyme can be phosphorylated by P_i

Methods

Uniformly labeled [G-^{3}H]ouabain was purchased from Amersham-Searle, [γ-^{32}P]ATP was synthesized enzymatically by the method of Post and Sen [18]

Plasma membranes rich in (Na^+ + K^+)-ATPase were prepared from the red outer medulla of rabbit or sheep kidney [19] by the method of Post and Sen [20]. These membranes were also extracted with 2 M NaI by a modification of the procedure of Nakao et al [21,22]

Rabbit kidney membranes (2-3 mg of membrane protein/ml) were incubated at 37°C in the presence of 20 mM NaCl, 2 5 mM Tris EDTA, 2 5 mM Tris EGTA (ethyleneglycol bis(α-aminoethylether)*N*,*N*′-tetraacetic acid), 10 mM imidazole-HCl (pH 7 5) and 5 mM *N*-ethylmaleimide until (Na^+ + K^+)-ATPase activity declined to 10—20% of the original value This process usually took 20—30 min and followed pseudo-first order kinetics. For each new batch of membranes the time necessary for 80—90% inactivation was determined before treating the bulk of membranes with *N*-ethylmaleimide This procedure assured reproducibility Reaction of the membranes with *N*-ethylmaleimide was stopped by 5 vol of ice-cold 10 mM imidazole HCl (pH 7 5) containing 10 mM 2-mercaptoethanol Subsequently the membranes were washed three times with 10 mM imidazole HCl (pH 7.5) by repeated centrifugation and resuspension In spite of protective ligands, 20—50% of Na^+ dependent phosphorylation was also inhibited, particularly when higher concentrations of *N*-ethylmaleimide (up to 10 mM) were employed in some experiments

Oligomycin was dissolved in acetone and dried to the bottom of the test tube under a stream of air before adding an aliquot of membranes Membranes were incubated with oligomycin at 0°C for 30 min before the addition of other ligands Oligomycin was used only in experiments at 0°C

For measuring total ATPase-activity, one ml of reaction mixture contained 100 μmol of NaCl, 20 μmol of KCl, 3 μmol of $MgCl_2$, 3 μmol of Tris ATP, 20 μmol of imidazole HCl (pH 7 4) and 5—15 μg of membrane protein For measuring (Na^+ + K^+)-insensitive ATPase one ml of the reaction mixture contained 3 μmol of $MgCl_2$, 3 μmol of Tris ATP, 1 μmol of ouabain, 20 μmol of imidazole HCl (pH 7 4) and 5—15 μg of membrane protein Inorganic phosphate liberated from ATP in both mixtures was measured by the procedure of Goldenberg and Fernandez [23] (Na^+ + K^+)-ATPase was taken as the difference in liberated phosphate between the two mixtures One unit of enzyme splits one μmol of ATP per min Protein was measured by the method of Miller [24]

The membranes were phosphorylated with [γ-^{32}P]ATP essentially as described by Post and Sen [18]. One ml of the reaction mixture usually contained either 100 μmol of NaCl, 1 μmol of $MgCl_2$, 5—50 nmoles of [γ-^{32}P]-ATP (200 cpm/pmol), 20 μmol of imidazole HCl (pH 7 4) and an aliquot of membranes (1 0—2 0 mg of membrane protein), or 100 μmol of KCl in place of NaCl in an otherwise identical mixture Specific phosphorylation of (Na^+ + K^+)-ATPase refers to the difference in the amount of phosphoprotein between these two mixtures The reaction was stopped with 25 ml of ice-cold 5% trichloroacetic acid containing 10 mM of phosphoric acid, the mixture was

filtered through Millipore-filter (pore size 0 45 μm) and the precipitated membranes were washed on the filter with 90 ml of ice-cold 30 mM HCl Phosphorylation reached steady-state at 0°C in about 4 s [3]

Before phosphorylation with $^{32}P_i$, the plasma membranes were washed at 0°C three times with a solution containing 1 mM $MgCl_2$ and 20 mM imidazole HCl (pH 7 4) to eliminate any Na^+ absorbed to the membranes since Na^+ would inhibit this phosphorylation [7] One ml of the reaction mixture contained 0 5 μmol of $MgCl_2$, 1 μmol of Tris $^{32}P_i$, (2000 cpm/pmol) 20 μmol of imidazole HCl (pH 7 4) and about 2 mg of membrane protein and other ligands as indicated in the legend to tables or figures To estimate non-specific phosphorylation either 20 mM NaCl was included in the above medium or the membranes were denatured by boiling for 3 min prior to addition to a Na^+ free medium Phosphorylation reached maximum at 0°C in 3—4 min, therefore 4 min was chosen as the minimal incubation time The reaction was stopped with 25 ml ice-cold 5% trichloroacetic acid containing 10 mM H_3PO_4 The membranes were sedimented, washed once with 30 ml of 20 mM HCl and then resuspended in 50 μl of 10 mM HCl Pepsin (Worthington) was dissolved also in 10 mM HCl and added to the membranes (1 mg pepsin per mg membrane protein) After digestion at room temperature for 30 min, the samples were centrifuged and peptides, released from the active site and labeled with ^{32}P, were separated from $^{32}P_i$ by high voltage electrophoresis [7] Conditions for electrophoresis were buffer 2% (v/v) formic acid (about pH 2), paper Whatman 3 MM, temperature 2°C, potential difference 35—40 volts per cm, time 90 min The ^{32}P-peptides were located by a thin-window Geiger counter, and also by viewing the paper under ultraviolet light Although the bulk of peptides seen are probably non-labeled contaminating peptides, viewing the paper under ultraviolet light, in addition to detecting the radioactivity, helps to separate neighboring spots derived from different samples The peptide spots were cut out, dried and the radioactivity was measured by liquid scintillation counting in a toluene-based mixture To correct for the hydrolysis of the phosphopeptides during digestion and electrophoresis an aliquot of the membranes was labeled with [γ-^{32}P]ATP, and the radioactivity in this sample was measured both by Millipore filtration as described earlier and by digestion and electrophoresis Estimated this way, the loss of radioactivity was 20—30% It was assumed for further calculations that all samples lost radioactivity to the same extent The validity of this procedure was proved by the good agreement among the level of phosphorylation from $^{32}P_i$ and from [γ-^{32}P]ATP (measured by filtration, without digestion) and the level of ouabain-binding (cf Tables I and V) To measure the amount of membranes from which the phosphopeptides were derived by peptic digestion, the residue was hydrolyzed with perchloric acid and the total phosphate content of the hydrolyzate was measured [25] The amount of membranes could be calculated on the basis of the total phosphate content of native enzyme which was not affected by acid-precipitation and was derived essentially from phospholipids since the amount of phosphoproteins and of absorbed inorganic phosphate was negligible [7]

Binding of [^{3}H]ouabain was measured according to Matsui and Schwartz [26] One ml of reaction mixture contained 50—200 μg of membrane protein, 1 nmol of [^{3}H]ouabain (unless otherwise indicated), 20 μmol of imidazole

HCl (pH 7 4) and one of four combinations of ligands (final concentrations) either 2 mM $MgCl_2$ or 2 mM $MgCl_2$ and 1 mM Tris P_i, or 100 mM NaCl, 2 mM $MgCl_2$ and 2 mM Na^+ ATP, or 5 mM Tris EDTA and 0 1 mM ouabain The last mixture served for the measurement of non-specific binding and this value was subtracted from the previous ones After incubation at the temperature and for the time indicated in the legends the membranes were sedimented, dissolved in alkali and the radioactivity was measured by liquid scintillation counting External standardization was used to correct for quenching.

Dissociation of ouabain from the enzyme was measured similarly to the method of Tobin and Sen [27]. First ouabain was bound to the membranes in the presence of one of the ligand combinations described previously After incubating at 37°C for 30 min binding was stopped and dissociation was started by adding one ml of 10 mM Tris EDTA prewarmed at 37°C. At different times samples were rapidly frozen in dry ice-acetone to block further dissociation At the end of the experiment all frozen samples were thawed at 0°C and the amount of bound ouabain was measured

All assays were done at least in triplicates and the average of these values is shown in the figures and tables

Results

One purpose of these experiments was to study the binding of ouabain to the ADP-sensitive phosphorylated from of (Na^+ + K^+)-ATPase The ADP-sensitive phosphoenzyme is formed in appreciable amounts only after poisoning the enzyme with oligomycin or *N*-ethylmaleimide Oligomycin is a reversible inhibitor that can be applied easily and does not affect Na^+-dependent phosphorylation Unfortunately at 37°C oligomycin seems to dissociate from the enzyme and does not inhibit more than about 50% of the activity [28—31] Oligomycin was therefore used only at 0°C. On the other hand *N*-ethylmaleimide is an irreversible inhibitor that forms a stable covalent bond with some SH groups of the enzyme Like some other inhibitors of (Na^+ + K^+)-ATPase (e g ouabain [12], F^- [32,33], Be^{2+} [34,35]), *N*-ethylmaleimide reacts differently with various ligand induced conformations of the enzyme it can inactivate preferentially either Na^+-dependent phosphorylation, or K^+-dependent dephosphorylation or both depending on the ligands By a double labeling procedure Hart and Titus [36] elegantly demonstrated that different combinations of ligands expose SH groups that are otherwise not accessible to *N*-ethylmaleimide For our experiments we ideally needed a preparation which formed 100% E_1-P, i e a phosphoenzyme completely insensitive to K^+ In the reaction mixture (cf Methods) EDTA and EGTA protected Na^+-dependent phosphorylation probably by chelating endogenous Ca^{2+} and/or Mg^{2+} [37] and the low concentration of Na^+ enhanced the reaction Inactivation of the enzyme by *N*-ethylmaleimide followed a single exponential (not shown) By the time 80—90% of the enzyme activity was inhibited (20—30 min at 37°C with 5 mM *N*-ethylmaleimide) Na^+ dependent phosphorylation declined only by 40—50% and the phosphoenzyme became almost completely insensitive to K^+ Such preparations were used primarily in this experiment though some experiments were repeated with oligomycin-treated membranes also The use of two chemically different inhibi-

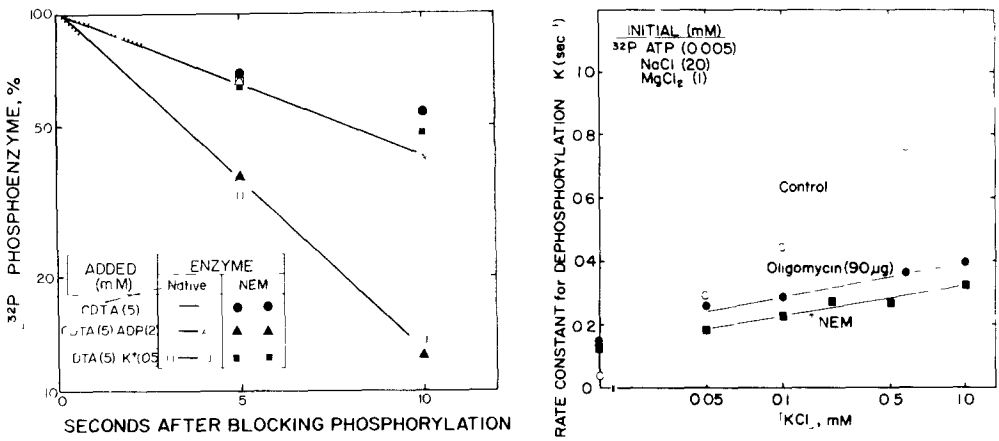

Fig 1 Effect of *N*-ethylmaleimide on dephosphorylation Native (0 32 mg protein) or *N*-ethylmaleimide treated (0 2 mg protein) rabbit kidney membranes were incubated at 0°C in a total volume of one ml, in the presence of 20 mM NaCl, 1 mM $MgCl_2$, 0 02 mM [γ-^{32}P] ATP and 10 mM imidazole-HCl (pH 7 5) Non-specific phosphorylation was measured by replacing NaCl with 20 mM KCl in the previous mixture This value was subtracted from each sample and the difference was plotted on the figure Phosphorylation was started at minus 5 s by adding [γ-^{32}P] ATP in 0 1 ml At 0 s the reaction was stopped in some samples with acid, the amount of [^{32}P] phosphoprotein was measured and this value was taken as 100% In other samples further phosphorylation was blocked and dephosphorylation was initiated with (1,2-cyclohexylenedinitrilo)tetraacetate (CDTA) (Tris-salt), CDTA + KCl or CDTA + ADP in concentrations indicated on the figure The measurements of [^{32}P] phosphoprotein is described in Methods

Fig 2 Effect of *N*-ethylmaleimide and oligomycin on the rate of dephosphorylation induced by K^+ The rate constant of dephosphorylation (k) at 0°C was calculated from graphs such as Fig 1 After phosphorylation reached a steady state in 5 s, dephosphorylation was started with 5 mM Tris-CDTA and KCl as indicated on the abscissa The control and oligomycin-treated samples contained 0 61 mg, the *N*-ethylmaleimide-treated samples contained 0 44 mg of membrane protein from rabbit kidney

tors helped to separate effects that might have been due to the specific structure of the inhibitors from effects that reflected properties of E_1-P itself

Fig 1 shows the characteristics of the phosphoenzyme in native and *N*-ethylmaleimide-treated membranes Both phosphoenzymes split spontaneously at a slow rate (k = 0 087 s^{-1} and 0 064 s^{-1} respectively) In the native enzyme dephosphorylation was enhanced significantly by 0 05 mM K^+ (k = 0 202 s^{-1}), but not by 2 mM ADP (k = 0 087 s^{-1}) On the contrary in *N*-ethylmaleimide-treated enzyme 2 mM ADP induced rapid dephosphorylation (k = 0 204 s^{-1}) while 0 05 mM K^+ had little effect (k = 0 074 s^{-1}) Fig 2 shows the K^+-dependence of the rate of dephosphorylation over a wider range of K^+ concentrations *N*-ethylmaleimide and oligomycin were equally effective in decreasing sensitivity to K^+

The turnover number and therefore the enzyme activity decreased after treatment with *N*-ethylmaleimide because of this slow rate of dephosphorylation even in the presence of K^+ (Table I) For the native enzyme the turnover number was about 10 000 if it was assumed that one mol of enzyme bound 1 mol of ouabain or formed 1 mol of phosphoenzyme [4] For the *N*-ethylmaleimide-treated enzyme the turnover number was 2570, specific activity of (Na^+ + K^+)-ATPase decreased by 87%, ouabain by about 50%, phosphorylation from ATP by about 45% One mol of phosphoenzyme bound 1 mol of ouabain

TABLE I

STOICHIOMETRIC RELATION AMONG ($Na^+ + K^+$)-ATPase ACTIVITY, PHOSPHORYLATION AND OUABAIN-BINDING OF NATIVE AND *N*-ETHYLMALEIMIDE ENZYME

Preparation of rabbit kidney membranes, treatment with *N*-ethylmaleimide and assay procedures were carried out as described in Methods All assays were performed in the presence of the indicated ligands and non-specific activities were subtracted The concentrations of substances were 2mM ATP for ($Na^+ + K^+$)-ATPase, 1 μM [^{3}H] ouabain for binding and 0 04 mM [γ-^{32}P] ATP for phosphorylation Ouabain was bound to the enzyme at 37° C

Assay	Unit	Ligands (mM)	Enzyme	
			Native	*N*-ethyl-maleimide-treated
($Na^+ + K^+$)-ATPase	μmol mg protein^{-1} min^{-1}	NaCl(100)KCl(10) $MgCl_2$(2)	1 5	0 2
Ouabain-binding	pmol mg protein^{-1}	$MgCl_2$(2)	61 8	12 1
		$MgCl_2$(2) P_i(1)	151 6	74 1
		NaCl(100) $MgCl_2$(2)ATP(2)	149 6	64 2
Phosphorylation	pmol mg protein^{-1}	NaCl(100)$MgCl_2$(1)	152 0	82 8

both in the native and in the *N*-ethylmaleimide-treated enzyme

If ouabain saturates the enzyme its binding follows pseudo-first order kinetics [38], i e. the free-binding sites disappear according to a single exponential (Fig 3) at least at the beginning of the reaction The rate constant for this process at a given concentration of ouabain was calculated from graphs such as Fig. 3 and was taken as the rate constant for association of ouabain with the enzyme. The rate of association was always fastest in the presence of Mg^{2+} and P_i and slowest in the presence of Mg^{2+} alone (Table II). This difference became even more pronounced after treating the membranes with *N*-ethylmaleimide or oligomycin both of which slightly but consistently slowed binding in the presence of Na^+, Mg^{2+} and ATP and accelerated binding in the presence of Mg^{2+} and P_i In all media, sheep kidney membranes bound ouabain about twice as fast as rabbit kidney membranes Both native and *N*-ethylmaleimide or oligomycin-treated enzyme formed type I complex more slowly than type II complex Since in a medium containing Na^+, ATP and Mg^{2+} the native enzyme formed E_2-P and the *N*-ethylmaleimide or oligomycin-treated enzyme formed E_1-P, these experiments also indicated that E_1-P bound ouabain somewhat more slowly than E_2-P did Slow conversion of E_1-P to E_2-P was not excluded though seems to be unlikely for the following reasons In the presence of Na^+, Mg^{2+} and ATP the rate of ouabain-binding by the *N*-ethylmaleimide-treated enzyme was distinguishable from that of the native enzyme and the disappearance of the free binding sites (as in Fig 3) could be described by a single exponential up to 40 minutes at 0° C If E_1-P had been converted into E_2-P during this period, disappearance of the free binding sites should not have followed a single exponential and the rate constant calculated at a later phase of the process (e g between 30 and 40 min) might have been more similar to that of the native enzyme Unfortunately small differences in these values

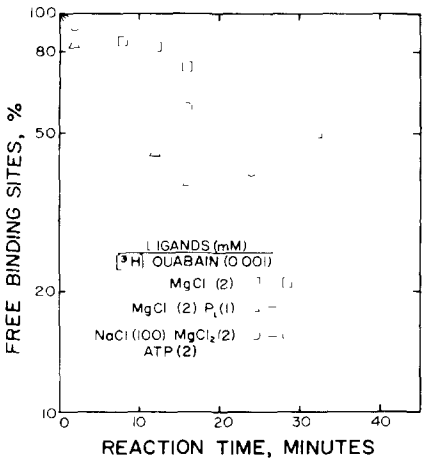

Fig 3 Time course of ouabain binding Ligands present during ouabain binding are shown on the figure In addition each sample contained in one ml 0 68 mg of membrane protein from rabbit kidney and 10 mM imidazole-HCl (pH 7 5) Non-specific binding (cf Methods) was subtracted from each value presented on the graph The total number of binding sites (100% on the graph) was measured by incubating some samples until binding reached a steady state (2 h at 0°C) Ouabain at the concentration used nearly saturated the binding sites whose dissociation constant was about 65 nM (cf Table IV) The measurement of ouabain-binding is described in Methods

might have escaped detection because of the relative insensitivity of the methods employed

Binding of ouabain is a relatively slow process [12,13,39] K^+ antagonizes the effect of ouabain by slowing the rate of ouabain binding without affecting the steady-state level of the enzyme-ouabain complex [40] K^+ may slow ouabain binding by decreasing the steady-state level of the phosphoenzyme since K^+ greatly accelerates dephosphorylation (cf Fig 1) As an alternative explanation there may be separate sites for K^+ to enhance dephosphorylation or to inhibit ouabain-binding *N*-ethylmaleimide-treated enzyme offers a model to

TABLE II

PSEUDO-FIRST ORDER RATE CONSTANTS OF FORMATION OF A MEMBRANE-OUABAIN COMPLEX AT 0°C

The pseudo-first order rate constants of association in the presence of 1 μM [^{3}H] ouabain were calculated from the rate of disappearance of free binding sites at 0°C (cf Fig 3) Ligands and inhibitors present during labeling are shown on the table

Species	Enzyme	Rate constant (k 10^4 s^{-1}) in the presence of (mM)		
		NaCl(100)-$MgCl_2$(2) ATP(2)	$MgCl_2$(2) P_i(1)	$MgCl_2$(2)
Sheep	native	7 5	7 9	2 9
	native + 90 μg oligomycin	6 0	8 7	3 0
Rabbit	native	3 2	3 7	1 4
	N-ethylmaleimide-treated	2 6	4 0	1 6

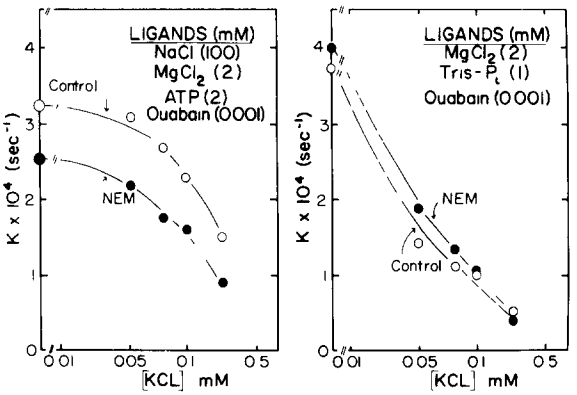

Fig 4 Effect of *N*-ethylmaleimide and K^+ on the rate of ouabain-binding at 0°C The concentrations of KCl and of other ligands present during ouabain-binding are shown on the abscissa and on the panel, resp The concentration of membrane protein (from rabbit kidney) was 0 4 mg/ml in the control, 0 78 mg/ml in the *N*-ethylmaleimide-treated sample The rate of disappearance of free binding sites was taken as pseudo-first order rate constant of association (k) and it was calculated from graphs such as Fig 3 The experimental procedure is described in Methods

test this hypothesis it forms a K^+-resistant phosphoenzyme from ATP which nonetheless binds ouabain Any direct effect of K^+ on ouabain-binding could therefore be separated from its effect on dephosphorylation Indeed, low concentrations of K^+ that did not increase dephosphorylation significantly (Fig 2) did slow the rate of ouabain-binding to the *N*-ethylmaleimide-enzyme as much as to the native one (Fig. 4) K^+ decreased the rate of binding both in the Na^+ + Mg^{2+} + ATP and in the Mg^{2+} + P_i medium and the K^+ induced changes were similar for the native and the *N*-ethylmaleimide-treated enzyme Thus, in the Na^+ + Mg^{2+} + ATP medium, K^+ could slow ouabain-binding to *N*-ethylmaleimide-treated enzyme without significantly changing the steady state level of the phosphoenzyme The experiments shown in Fig 2 and Fig 4 can be compared directly with each other because the same membrane preparation was used at the same temperature (0°C), only the substrate concentrations were modified for optimal phosphorylation and ouabain-binding Oligomycin (90 μg/ml), just as *N*-ethylmaleimide, did not affect the K^+ sensitivity of ouabain-binding (not

TABLE III

RATE CONSTANTS FOR DISSOCIATION OF THE ENZYME-OUABAIN COMPLEX AT 37°C

Dissociation rate constants were measured as described in Methods and were calculated from graphs such as Fig 5 These samples contained in one ml 0 23 mg of membrane protein from rabbit kidney, 1 μM [^{3}H]ouabain and other ligands as indicated

Ligands (mM)	Rate constant for dissociation (k 10^3 s^{-1})	
	Native enzyme	*N*-ethylmaleimide-treated enzyme
$MgCl_2$(2)	3 2	1 4
$MgCl_2$(2)P_i(1)	3 2	2 8
NaCl(100)$MgCl_2$(2) ATP(2)	4 2	10 5

TABLE IV

DISSOCIATION CONSTANT OF THE ENZYME-OUABAIN COMPLEX AT 37°C

The dissociation constant (K_s) was determined by measuring the steady state level of bound ouabain at different ouabain concentrations in the presence of the ligands indicated and by plotting the data according to Scatchard [41] Rabbit kidney membranes were used in all experiments

Ligands (mM)	Dissociation constant (K_s)	
	Native enzyme	*N*-ethylmaleimide-treated enzyme
NaCl(100)$MgCl_2$(2) ATP(2)	65 5 nM	142 6 nM
$MgCl_2$(2)P_i(1)	64 8 nM	58 6 nM

shown), thus the phenomenon did not depend on the chemical structure of the inhibitor employed for producing E_1-P Dissociation of the enzyme-ouabain complexes was measured at 37° C (half-time is 3—5 min) because at 0° C dissociation is very slow (half time is about 9 h [27]) The type I complex was less

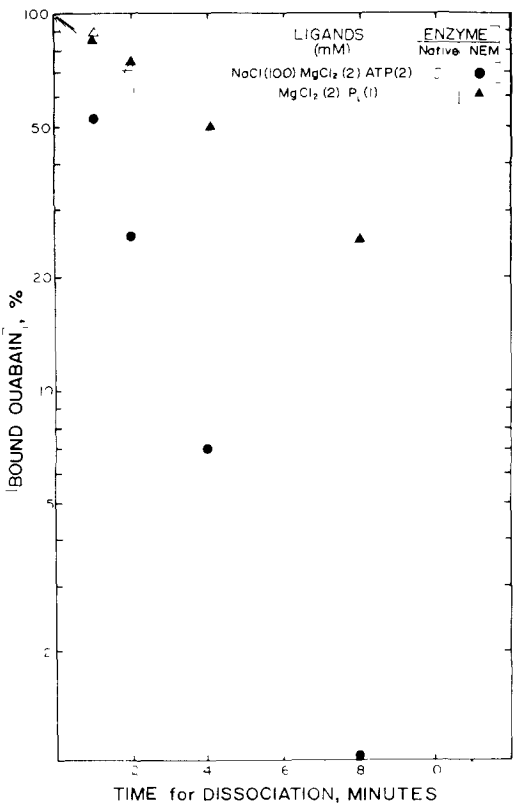

Fig 5 Effect of *N*-ethylmaleimide on the dissociation of the enzyme-ouabain complex at 37°C Ligands present during binding are shown on the graph In addition each sample contained in one ml 1 nmol of [^{3}H]ouabain and 0 48 mg of native or 0 88 mg of *N*-ethylmaleimide-treated membrane protein from rabbit kidney Ouabain-binding reached a steady state in 30 min at 37°C and this level of bound ouabain was taken as 100% on the graph The experimental procedure is described in Methods

stable than the type II complex both in the native and in the *N*-ethylmaleimide-treated enzyme (Fig. 5 and Table III) This difference in stability was even more pronounced in the *N*-ethylmaleimide-treated enzyme because dissociation of the type II complex was slightly slower and that of the type I complex about twice as fast as in the native enzyme (Table III) These changes were also reflected in the dissociation constant of the enzyme-ouabain complexes measured at 37°C and calculated from Scatchard plots [41] (Table IV) The simplest interpretation of these data is that *N*-ethylmaleimide decreased primarily the stability of the type I complex, i e ouabain formed a less stable complex with E_1-P than with E_2-P On the other hand, *N*-ethylmaleimide did not modify the properties of the type II complex to the same extent (cf e g the dissociation constants in Table IV)

It was surprising that P_i could enhance the formation of the type II complex in the presence of Mg^{2+} (Table II) in the *N*-ethylmaleimide enzyme This finding indirectly indicated that *N*-ethylmaleimide did not abolish phosphorylation by P_i in the presence of Mg^{2+} and therefore the *N*-ethylmaleimide-treated enzyme formed E_1-P from ATP and probably E_2-P from P_i Earlier Siegel et al [15] did not observe phosphorylation of electroplax membranes by P_i after *N*-ethylmaleimide treatment We reinvestigated this phosphorylation by P_i of the *N*-ethylmaleimide-treated enzyme to exclude the remote possibility that, unlike in the native enzyme, P_i supported ouabain-binding to *N*-ethylmaleimide-treated enzyme without prior phosphorylation P_i did phosphorylate the *N*-ethylmaleimide-treated enzyme and this phosphorylation was inhibited by Na^+ just as in the native enzyme (Table V) On the other hand phosphorylation by ATP of both native and *N*-ethylmaleimide-treated enzyme required Na^+, and Na^+ could not be replaced by K^+ The discrepancy between these and earlier findings [15] can probably be resolved by pointing out some technical differences We could measure phosphorylation by P_i only if the membranes were digested and the ^{32}P-labeled peptides from the active site of (Na^+ + K^+)-ATPase were separated from $^{32}P_i$ by electrophoresis. Repeated washings with acids [15] were not always sufficient to rid the membranes of $^{32}P_i$ that was not bound covalently but made the measurement of specific labeling impossible It is important to note that, within the accuracy of the procedures, the same

TABLE V

LIGAND SENSITIVITY OF PHOSPHORYLATION

Rabbit kidney membranes were phosphorylated with [γ-^{32}P] ATP or $^{32}P_i$ at 0°C as described in Methods Non-specific phosphorylation was not subtracted from the values shown (Na^+ + K^+)-ATPase activity was 1 62 units/mg protein for the native and 0 20 units/mg protein for the *N*-ethylmaleimide-treated enzyme

Ligands (mM)	[32P] Phosphoenzyme (pmol mg protein^{-1})	
	Native enzyme	*N*-Ethylmaleimide-treated enzyme
$^{32}P_i$(1)$MgCl_2$(0 5)	179 5	80 5
$^{32}P_i$(1)$MgCl_2$(0 5)NaCl(100)	7 9	2 6
[^{32}P] ATP(0 02)$MgCl_2$(1 0)NaCl(100)	155 6	74 1
[^{32}P] ATP(0 02)$MgCl_2$(1 0)KCl(100)	1 5	0 2

amount of phosphoenzyme was formed from P_i as from ATP, and both types of phosphoenzyme bound one mole of ouabain both in the native and in the *N*-ethylmaleimide-treated enzyme (Table I) The *N*-ethylmaleimide-treated enzyme formed more phosphoenzyme from either ATP or P_i than expected on the basis of the remaining $(Na^+ + K^+)$-ATPase activity and the normal turnover (10 000/min) of a native enzyme (cf legend to Table V) These facts indicated that indeed the *N*-ethylmaleimide-treated enzyme molecules were interacting with ATP, P_i and ouabain and not a small fraction of native enzyme molecules that had not reacted with *N*-ethylmaleimide Oligomycin (90 μg/ml) did not abolish phosphorylation by P_i either and this phosphorylation could also be inhibited by Na^+ (not shown) (Further study of phosphorylation by P_i of the *N*-ethylmaleimide or oligomycin-treated enzyme is in progress and will be reported later)

Discussion

Formation of the type I complex

Type I complex between enzyme and ouabain is formed in the presence of Na^+, Mg^{2+} and ATP In the presence of these ligands the native enzyme forms a K^+-sensitive phosphoenzyme (E_2-P), the *N*-ethylmaleimide- or oligomycin-treated enzyme forms an ADP-sensitive phosphoenzyme (E_1-P) (see also Introduction) Formation of a type I complex by the enzyme treated with *N*-ethylmaleimide or oligomycin therefore shows that E_1-P can bind ouabain This phenomenon was described earlier [15,16,17] though Post et al [17] could observe this binding only at 23°C but not at 0°C The lack of ouabain binding to an *N*-ethylmaleimide treated enzyme in their experiments was probably due to the short incubation time (10—50 s in contrast to 20—60 min in our experiments) By its kinetic characteristics (rate constant of association and of dissociation, dissociation constant, cf Tables II, III, IV), the (E_1-P)-ouabain complex can be clearly distinguished from the (E_2-P)-ouabain complex The most prominent difference was the faster rate of dissociation of the (E_1-P)-ouabain complex (Table IV) The obvious difference between (E_1-P)-ouabain and (E_2-P)-ouabain, formed in the presence of Na^+, Mg^{2+} and ATP, also showed that the so-called type I complex is not necessarily a homogeneous species but may include both of these complexes even in the native enzyme

The role of E_1-P in the reaction mechanism of $(Na^+ + K^+)$-ATPase is still not resolved It has been assumed, but never proved, that E_1-P is precursor of E_2-P with the equilibrium shifted toward the formation of predominantly E_2-P [8,9] This idea was supported by the demonstration of some E_1-P that could react with deoxy-ADP in the native enzyme also [42] E_1-P also accumulated upon lowering the Mg^{2+} concentration and without adding any inhibitor but only if the enzyme was prepared from the electroplax of the electric eel and not from mammalian tissues [9] In contrast to the previous interpretation it was suggested [10] that E_1-P may represent an alternative pathway of the reaction and may not occur in the native enzyme Our results do not answer this problem conclusively but tend to support the second hypothesis Since ouabain should stabilize ("trap") E_1-P even in a native enzyme, if E_1-P were a mandatory intermediate the kinetic properties of the type I complex should

then be identical in both the native and the *N*-ethylmaleimide-treated enzyme, contrary to what was found in these experiments This apparent lack of (E_1-P)-ouabain in the native enzyme, however, does not rule out that some E_1-P was formed because (a) if the conversion of E_1-P into E_2-P is much faster than the formation of the (E_1-P)-ouabain complex, ouabain cannot "trap" minute amounts of (E_1-P); and (b) (E_1-P)-ouabain may not be detected by kinetic measurements alone in the presence of large excess of (E_2-P)-ouabain

Formation of type II complex

Both the native and the *N*-ethylmaleimide- or oligomycin-treated enzymes bound some ouabain in the presence of Mg^{2+} alone, however, the addition of P_i greatly enhanced both the rate and the steady-state level of ouabain-binding (Table I, II) P_i was bound covalently to the active site of *N*-ethylmaleimide-treated enzyme also because (a) labeled peptides liberated by peptic digestion and separated by high voltage electrophoresis have the same mobility as those formed by the native enzyme, (b) phosphorylation by P_i was inhibited by Na^+ (Table V) both in the native and in the *N*-ethylmaleimide-enzyme, and finally (c) the same amount of phosphoenzyme was formed from P_i as from ATP P_i, therefore, enhanced ouabain-binding by phosphorylating the active center rather than by non-covalent binding to some allosteric site Na^+, which inhibited phosphorylation by P_i, abolished the enhancement of ouabain-binding by P_i also Dahms and Boyer [43] showed that oligomycin did not inhibit the exchange of oxygen between P_i and water which involves phosphorylation of (Na^+ + K^+)-ATPase. It came as a surprise, however, that *N*-ethylmaleimide did not inhibit phosphorylation by P_i either (Siegel et al [15] did not observe this phosphorylation possibly for technical reasons as explained in the Results) Obviously the SH group(s) that *N*-ethylmaleimide blocked under the specific conditions of these experiments were not necessary for phosphorylation by P_i These experiments therefore directly demonstrated that the *N*-ethylmaleimide-treated enzyme forms E_1-P from ATP and E_2-P from P_i

From the analysis of ATP binding [22,44] and phosphorylation by P_i [7] it became evident that the dephosphoenzyme exists at least in two reactive states one form has high affinity for ATP and Na^+, and can be phosphorylated by ATP (E_1), another form has low affinity for ATP, binds K^+ and can be phosphorylated by P_i (E_2) Phosphorylation by P_i requires conversion of E_1 to E_2 and this conversion is apparently inhibited by Na^+ [7] *N*-ethylmaleimide did not inhibit this conversion since the *N*-ethylmaleimide-treated enzyme could be phosphorylated by both ATP and P_i

Effects of K^+ on phosphorylation and ouabain-binding

K^+ stimulates (Na^+ + K^+)-ATPase at multiple sites with cooperative interactions among these sites [45] In addition K^+ accelerates dephosphorylation [4], slows ouabain-binding [13,40], stimulates the splitting of *p*-nitrophenylphosphate or acetylphosphate [1,2] modifies inhibition by *N*-ethylmaleimide, F^- [32,33] and Be^{2+} [34,35] and stabilizes some form of the enzyme-ouabain complex [46–48], though this final effect was not observed in intact cells [40] K^+ probably brings about these effects at sites with different affinity [49] In our experiments the rate of dephosphorylation of native phospho-

enzyme was doubled by as low as 0.015 mM K^+ but only 0 29 mM K^+ decreased the rate of formation of type I complex by a factor of two (0 35 mM K^+ for type II complex) In the case of *N*-ethylmaleimide enzyme, dephosphorylation was almost completely insensitive to K^+ (Fig 1) but formation of the enzyme-ouabain complexes could be inhibited by K^+ (two-fold decrease in the rate of formation was brought about 0 21 mM K^+ for type I complex and by 0 38 mM K^+ for type II complex). These facts can be interpreted so that *N*-ethylmaleimide or oligomycin blocked some high affinity sites at which K^+ normally enhances dephosphorylation but these inhibitors did not affect some moderate affinity sites at which K^+ slows the rate of ouabain-binding In support of previous kinetic evidence our own experiments showed directly that these two classes of K^+ sites (with high and moderate affinity) can be inhibited independently and are therefore probably separate Different inhibitors (e g *N*-ethylmaleimide or oligomycin) may thus promote the formation of E_1-P by modifying these high affinity sites or by blocking the access of K^+ to them

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