The Reaction of Sulphhydryl Groups of Sodium and Potassium Ion-activated Adenosine Triphosphatase with N-Ethylmaleimide

THE RELATIONSHIP BETWEEN LIGAND-DEPENDENT ALTERATIONS OF NUCLEOPHILICITY AND ENZYMATIC CONFORMATIONAL STATES*

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The reaction between N-ethylmaleimide and (Na+ + K+)-ATPase, performed under ligand conditions which produce each of the kinetic states of the enzyme and their associated conformational forms, was examined through an analysis of the inhibition of enzymatic activity and the incorporation of radiolabeled reagent into the enzyme. The inactivation reactions displayed pseudo-first order kinetics with respect to the concentration of active enzyme, indicating that the loss of activity is associated with the alkylation of a unique sulphhydryl group. In the absence of enzyme phosphorylation, the nucleophilicity of this sulphhydryl group is affected primarily by the nature of the monovalent cation present and does not correlate with the conformational state.

A method for determining the actual concentration and specific radioactivity of radiolabeled N-ethylmaleimide during the reaction with (Na+ + K+)-ATPase was developed, allowing the measurement of the total reactive sulphhydryl groups of native (Na+ + K+)-ATPase under conditions identical with those of the inactivation studies. The labeling of the enzyme complex is associated almost exclusively with the large polypeptide, which contains four sulphhydryl groups which react stoichiometrically and rapidly with N-ethylmaleimide under all conditions. The nucleophilicity of the fourth sulphhydryl group is governed by the conformational state of the enzyme, but the alkylation of this residue does not result in loss of enzymatic activity.

Sodium and potassium ion-activated adenosine triphosphates is the enzyme which catalyzes the hydrolysis of MgATP coupled to the active transport of Na+ and K+ across cell membranes (1, 2). The larger of the two polypeptides

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1 The abbreviations used are: (Na+ + K+)-ATPase, sodium and potassium ion-activated adenosine triphosphatase; SDS, sodium dodecyl sulfate; CDTA, trans-1,2-diaminocyclohexanetetraacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); ESC, S-(N-ethylsuccinimidyl)-L-cysteine.
under conditions which maintain the native enzyme. The nucleophilicity of one of these is affected by the nature of the ligands present, while that of another is controlled by conformational state. The alkylation of only the former leads to a conformational change in the enzyme. It is also shown that conformational states exist in addition to an inactive state. It is also shown that conformational states exist in addition to an inactive state.

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Isolation and Determination of Specific Radioactivity of the Large Polypeptide—In all experiments, some samples were incubated with SDS-polyacrylamide gels prepared with 5% acrylamide and 0.14% bisacrylamide according to the method of Weber and Osborn (34). Lyophilized samples, containing SDS from the previous dialysis, were dissolved in water and made 5% in 2-mercaptoethanol.

Three different methods which gave identical results within experimental error were used for the quantitation of radiolabeled N-ethylmaleimide covalently bound to the large polypeptide. The first method utilized preparative gel electrophoresis. A protein sample (200 μg) was applied to a gel which was submitted to electrophoresis and then immediately scanned at 280 nm on a Zeiss PMQII spectrophotometer fitted with a linear transporting chamber. The region containing the α polypeptide was cut out of the gel in 5 to 10 slices (1 mm) and eluted with three changes (2 ml) of 50 mM ammonium bicarbonate buffer, pH 7.8, 0.1% SDS, 25 mM 2-mercaptoethanol at 37 °C over 24 h. Equivalent portions of blank gels, as controls, were also eluted. These samples were pooled, passed through a Millipore filter (0.45 μm), lyophilized, and redissolved in 200 μl of water. Triplicate aliquots were taken for both liquid scintillation and quantitative amino acid analysis. For the latter, norleucine was added as an internal standard and at least 10 amino acids were used to calculate protein concentration, correcting for the background in the control (α + β + γ) and relative amount of each amino acid was calculated with the published composition of the α polypeptide (35). The molar ratio of incorporation was calculated using the independently determined specific radioactivity of the N-[14C]ethylmaleimide, the specific radioactivity of the α polypeptide, and the value of 121,000 for the molecular weight of the α polypeptide (36). The relative amount of each amino acid was calculated with the published composition of the α polypeptide. In addition to the direct method of measurement just described, a second method of quantitation was also used. Gels were stained for protein with Coomassie brilliant blue, scanned at 500 nm, and the values determined by the two other methods were normalized to the scanned areas, and the molar ratios of N-ethylmaleimide incorporated into each polypeptide, for all experimental conditions, were calculated from the normalized values obtained for the standards and their known molar ratios. The relative values among the three standards in each experiment always agreed closely with expectation.

Inhibition of (Na+ + K+)-ATPase by N-Ethylmaleimide—Incubation of SDS-purified and nucleotide-free (Na+ + K+)-ATPase with 5 mM N-ethylmaleimide at pH 7.5, 37 °C, and in the presence of various combinations of ligands progressively inhibits its enzymatic activity. The combination and concentration of the ligands chosen here represent conditions in which saturation of a given ligand site or sites is ensured and which, as a result, produce a kinetic state and poise a majority of the enzyme in a discrete equilibrium conformation. Kinetic examinations of this inactivation, over periods of 30 to 60 min, were performed. The enzyme was preincubated in the presence or absence of ligands, and the reaction was initiated by the addition of N-ethylmaleimide. Aliquots were withdrawn at 5-min intervals, the reaction quenched with 2-mercaptoethanol, and the samples were assayed for (Na+ + K+)-ATPase activity. In all cases, except for that in which oligomycin was present, the inactivation reactions of the various conformational forms of the enzyme displayed pseudo-first order kinetics with respect to the inactivation of active enzyme, indicating that the loss of activity is associated with the alkylation of a unique sulfhydryl group.

A comparison of the respective rate constants for the loss of enzymatic activity, therefore, permits this sulfhydryl group to take the role of a reporter of the conformation. Each of the pseudo-first order rate constants was calculated, and they are presented in Table I in units relative to the slowest reaction. These results demonstrate that, in the absence of enzyme phosphorylation, the nucleophile of the sulfhydryl group involved in the loss of enzymatic activity is governed mainly by the nature of the cation present and does not correlate with the conformational changes associated with each kinetic state of the enzyme. In the presence of K+, the rate of the alkylation of this sulfhydryl is about 2.5 to 5 times the rate in the presence of Na+ under the same conditions. Control experiments, involving alkylation in the presence of Na+, K+, or Tris at ionic strengths equivalent to that of 100 mM Na+, proceed at rates identical with those presented in Table I for the other concentrations of these cations. This observation indicates that the differences in conformational change between the two cation substrates cannot be attributed to an ionic strength effect. The addition of Mg2+ to an otherwise equivalent solution also seems to decrease the rate by about 30%. It can also be seen that the binding of ATP in the absence of these cations, decreases the inactivation rate to values characteristic of alkylation in the presence of Na+.

The phosphorylation of the enzyme in the presence of either Na+, Mg2+, and ATP, or Mg2+, P, and strophanthidin, results in the E-P kinase state (4, 7, 44, 45). Under either of these conditions, the enzyme activity is the least susceptible to alkylation, with 80% usually remaining after 30 min. If Mg2+ is omitted from the incubation with strophanthidin and P, the loss of activity is substantial, suggesting that actual phosphorylation is necessary for this protection of the critical sulfhydryl group.

When a less purified preparation of (Na+ + K+)-ATPase, deoxycholate-treated zonal gradient microsomes (28, 29) is incubated with N-ethylmaleimide in the presence of an ATP regeneration system under conditions required for Na+-dependent phosphorylation, the same slow rate of inactivation 1

It will be assumed that the products of the alkylation of the enzyme are ethylmucycinidocysteine residues within the polypeptide chain.
that the final concentrations were NaCl, 7.5 M MgCl₂, and operating system consisting incubated at withdrawn at first order rate constants after the addition of N-ethylmaleimide, with subsequent aliquots is observed. If components of the regeneration system are ATPase activity, ATP, when present alone, is rapidly hydro-

imated enzyme preparation contains substantial Mg²⁺-stimulated and strophanthidin-insensitive ATPase activity, ATP, when present alone, is rapidly hydrolyzed and the enzyme cannot maintain the phosphorylated conformation. This is supported further by the observation that phosphorylation by ATP of SDS-purified enzyme, a preparation containing very little Mg²⁺-stimulated ATPase activity, gives identical results regardless of the presence or

TABLE I
Relative rates of the inactivation of (Na⁺ + K⁺)-ATPase with
N-ethylmaleimide

| Conditions                                      | Kinetic state | Refs. | kₜ | No. of measurements |
|------------------------------------------------|---------------|-------|----|---------------------|
| K⁺ + ATP + CDTA                                | E₁            | 11-13, 15-17 | 7.1 | (7)                 |
| K⁺ + ATP + Mg²⁺                                | E₁            | 16, 17  | 5.0 | (2)                 |
| Pᵢ + strophanthidin                           |               |        | 5.0 | (2)                 |
| (−Me⁺)²⁺                                      |               |        |     |                     |
| Enzyme alone (−CDTA)                          | E₁            | 17     | 4.9 | (10)                |
| Enzyme alone (−CDTA)                          | E₁            | 17     | 4.9 | (9)                 |
| K⁺ + CDTA                                     | E₁, E₂      | 13-17  | 4.8 | (4)                 |
| K⁺ + Mg²⁺                                     | E₁, E₂      | 12, 13, 16, 37 | 4.8 | (4)     |
| Na⁺ + ATP + Mg²⁺ + re-generation system       | (−phosphoenolpyruvate or pyruvate kinase)²⁺      | 4.5 | (2)   |
| CDTA                                          | E₁, E₂      | 17     | 2.8 | (12)                |
| ATP + CDTA                                    | E₁           | 11, 12, 16, 37 | 2.4 | (2)                 |
| Na⁺ + CDTA                                    | E₁           | 17     | 2.2 | (6)                 |
| Na⁺ + ATP + CDTA                              | E₁           | 11-13, 15-17 | 2.1 | (7)                 |
| 0.5 M Na⁺ + ATP + Mg²⁺                        | E₁, ~P      | 39, 40  | 1.6 | (5)                 |
| Na⁺ + ATP + Mg²⁺ + K⁺                        | E₁, ~P      | 8, 41, 42 | 1.0 | (10)                |
| + oligomycin²⁺                                 |               |        |     |                     |
| ATP + Mg²⁺                                    | E₁           | 12, 43  | 1.6 | (2)                 |
| Na⁺ + ATP + Mg²⁺ + re-generation system       | E₁, E₂      | 11, 13, 15-17 | 1.0 | (5)                 |
| Na⁺ + ATP + Mg²⁺ + re-generation system²⁺     | E₁, E₂      | 11, 13, 15-17 | 1.0 | (5)                 |
| Na⁺ + ATP + 50 mM Mg²⁺                        | E₁, E₂      | 11, 13, 15-17 | 1.0 | (8)                 |
| Na⁺ + ATP + Mg²⁺                             | E₁, E₂      | 11, 13, 15-17 | 1.0 | (4)                 |
| Pᵢ + Mg²⁺ + strophanthidin                   | E₁, E₂      | 7, 16, 44 | 1.0 | (4)                 |
| Pᵢ + Mg²⁺ + strophanthidin²⁺                 | E₁, E₂      | 7, 16, 44 | 1.0 | (4)                 |

²⁺ These conditions represent experimental controls; the enzyme conformation has not been determined in the presence of these ligands.

³ Protection is observed to the same degree as Na⁺ + ATP + Mg²⁺; however, the inactivation does not exhibit pseudo-first order kinetics.

is observed. If components of the regeneration system are omitted, however, substantial loss of enzymatic activity oc-

absence of the ATP regeneration system. With this preparation of enzyme, only about 15% of the ATP is hydrolyzed during the incubation under these conditions. It is likely that earlier studies of the alkylolation (18, 20, 23, 46) of the ATP-dependent phosphorylated state, utilizing impure preparations of enzyme, were complicated by losses of ATP similar to those described here.

High Na⁺ concentration (0.5 M) in addition to Mg²⁺ and ATP, conditions which produce the E₁ ~ P kinetic state (39, 40, 47, 48), produces an enzyme form only slightly more nucleophilic than the E₂-P. Oligomycin also is thought to stabilize the E₂-P form of the enzyme in the presence of Na⁺, Mg²⁺, and ATP (8, 41, 42). Following alkylolation in the presence of oligomycin, the (Na⁺ + K⁺)-ATPase activity remaining after 30 min was the same as that found in the E₂-P form, but the kinetics was not pseudo-first order. Therefore, a quantitative comparison of the rate constants cannot be made in this case. Nevertheless, the sulphydryl residue whose alkylation leads to inactivation of the enzyme displays almost equivalent nucleophilicity in both of the phosphorylated forms of the enzyme, E₁ ~ P and E₂-P, and therefore can detect no differences between them.

The experimental results obtained with other reporters of ligand-stabilized conformations are usually interpreted in terms of two basic forms of the enzyme, E₁ and E₂, with the assumption that E₁ and E₂-P are conformationally equivalent, as well as E₁ and E₂-P. The results reported here, however, demonstrate that the conformational responses detected by this activity-associated sulphydryl group depends on ligand conditions which do not strictly correlate with the stabilization of either the E₁ or E₂ forms.

Determination of Concentration and Specific Radioactivity of Radionabeled N-Ethylmaleimide—The amount of N-ethylmaleimide incorporated by (Na⁺ + K⁺)-ATPase while the enzyme is maintained in different ligand-stabilized kinetic states is a measure of the total accessible sulphydryl groups. A successful evaluation of this quantity potentially could indicate structural changes affecting sulphydryl groups other than the one associated with the loss of activity which was just described. Indeed, earlier experiments (18), which followed the incorporation of N-ethylmaleimide into (Na⁺ + K⁺)-ATPase under various ligand conditions, suggested that this might be the case. At the outset of the present experiments, however, it became clear that there are serious difficulties involved in the estimation of the concentration and specific radioactivity of N-ethylmaleimide due to the tendency of the reagent to polymerize, hydrolyze, or evaporate. Therefore, an accurate method for determining the actual concentration and specific radioactivity of active, monomeric, radionabeled N-ethylmaleimide during the reaction with (Na⁺ + K⁺)-ATPase was necessary. The method developed is based on the reaction of N-ethylmaleimide with L-cysteine to form ethylsuccinimidocysteine (33). The two diastereoisomeric products are ninhydrin-positive and can be resolved by cation exchange chromatography on an amino acid analyzer. The molar concentration of ESC is quantitatively determined by the ninhydrin reaction and the effluent is collected to measure the associated radioactivity. From these two quantities the actual specific radioactivity of N-ethylmaleimide can be determined. Furthermore, since the reaction converts the N-ethylmaleimide quantitatively into ESC, the true molar concentration of N-ethylmaleimide in the original sample is also established.

This assay was used to monitor the fate of the N-ethylmaleimide during various steps of the enzyme-labeling procedure (Table II). These manipulations include transfer of the radio-

J. Winslow, unpublished observation.
TABLE II
Losses and changes in specific radioactivity of N-ethylmaleimide resulting from preparation of alkylation mixtures

A stock solution was prepared by transferring radioactive N-ethylmaleimide from the pentane solution supplied by the manufacturer to histidine/sucrose buffer. To this stock solution (Na\(^+\) + K\(^+\)-ATPase (1 to 3 mg ml\(^{-1}\)) was added to initiate an alkylation experiment. Samples were removed from the stock solutions or from the alkylation mixtures immediately following the addition of enzyme for direct determinations of the molar concentration and specific radioactivity of the N-ethylmaleimide in each of these solutions by the reaction of radiolabeled N-ethylmaleimide with L-cysteine. The values tabulated are calculated from these assays, the specifications listed by the manufacturer, or the actual mass of crystalline reagent used in the experiments.

| Step in procedure | \(1^\circ\) | \(2^\circ\) | \(3^\circ\) | \(4^\circ\) |
|-------------------|-----------|-----------|-----------|-----------|
| 1. nmol radiolabeled N-ethylmaleimide in pentane transferred to buffer (calculated from manufacturer's specifications) | 910 | 239 | 230 | 1200 |
| 2. \(\mu\)Ci radiolabel transferred to buffer (direct determination) | 4.3 | 37.7 | 37 | 10 |
| 3. nmol radiolabeled N-ethylmaleimide dissolved as intact monomer in buffer after pentane evaporation (direct determination) | 280 | 150 | 108 | 270 |
| 4. Per cent recovery of intact, monomeric N-ethylmaleimide following transfer (steps [3 + 1] \(\times\) 100) | 31 | 64 | 47 | 23 |
| 5. nmol nonradioactive N-ethylmaleimide added (calculated from mass of crystalline solid) | 370 | 850 | 850 | 600 |
| 6. Total nmol of intact, monomeric N-ethylmaleimide, radioactive and nonradioactive, present in final stock solution (direct determination) | 380 | 810 | 710 | 550 |
| 7. Per cent of total nmol of N-ethylmaleimide added, radioactive and nonradioactive, recovered as intact monomeric reagent in stock solution (steps [6 + (1 + 5)] \(\times\) 100) | 30 | 74 | 65 | 30 |
| 8. \(\mu\)Ci radiolabeled, monomeric intact N-ethylmaleimide present in final enzyme alkylation mixture (direct determination) | 1.12 | 13.5 | 6.3 | 0.83 |
| 9. Per cent recovery of \(\mu\)Ci from supplier's container to experimental mixture (8 + 2) \(\times\) 100) | 26 | 36 | 17 | 8 |
| 10. Final specific radioactivity of predicted for alkylation mixture based on total \(\mu\)Ci and total nmol added (12 + (1 + 5)]) | 3.3 | 35 | 35 | 5.6 |
| 11. Final specific radioactivity of intact monomeric N-ethylmaleimide in alkylation mixture (direct determination) | 3.0 | 16.7 | 8.9 | 1.48 |

\(^a\) \(N\-[\text{H}^3]\text{H}\)ethylmaleimide.
\(^b\) \(N\-[\text{C}^4]\text{C}\)ethylmaleimide.
\(^c\) mCi mmol\(^{-1}\).

labeled N-ethylmaleimide from pentane to reaction buffer, removal of the pentane, the addition of concentrated, nonradioactive N-ethylmaleimide to achieve the desired final concentration of the stock reagent solution, and the addition of enzyme to the stock reagent solution to initiate the alkylation reaction. As can be seen (Table II), there is a loss of both radioactive and nonradioactive N-ethylmaleimide during the preparation of the stock solution as well as when the stock solution is mixed with the enzyme. This variability in recovery illustrates the complete impossibility of calculating the specific radioactivity indirectly and emphasizes the absolute necessity for a quantitative assay in studies which rely upon the specific radioactivity during sulfhydryl determinations with radiolabeled N-ethylmaleimide.

Alkylation of (Na\(^+\) + K\(^+\))-ATPase with Radiolabeled N-Ethylmaleimide—Two preparations of (Na\(^+\) + K\(^+\))-ATPase, SDS-purified enzyme (30) and supernatant enzyme (26), were used in these experiments. Both contain no polypeptides other than the \(\alpha\) and \(\beta\) chains of the enzyme. Samples from the preparations were incubated for 30 min with either \(N\-[\text{H}^3]\) ethylmaleimide or \(N\-[\text{C}^4]\)ethylmaleimide whose specific radioactivity and concentration has been measured by the ESC assay. The reaction mixtures were quenched with 2-mercaptoethanol, denatured with SDS, and the polypeptides separated by electrophoresis on 5% SDS-polyacrylamide gels. The gels were scanned at 280 nm, and the areas corresponding to the \(\alpha\) and \(\beta\) chains were sliced and counted. Alternatively, the gels were stained, scanned at 500 nm, and sliced and counted. Typical results are shown in Fig. 1. It can be seen that the great majority of the radioactivity is associated with the \(\alpha\) subunit.

![Image of Fig. 1. Distribution of radiolabeled N-ethylmaleimide reacted with (Na\(^+\) + K\(^+\))-ATPase. SDS-purified enzyme, \(A\), and supernatant enzyme, \(B\), (1 to 3 mg ml\(^{-1}\)) were labeled in histidine/sucrose buffer in the absence of ligands by the addition of \(N\-[\text{H}^3]\) ethylmaleimide and \(N\-[\text{C}^4]\)ethylmaleimide, respectively. Aliquots were removed immediately after the addition of the reagent for direct determinations of the final molar concentration and specific radioactivity of \(N\-[\text{H}^3]\)ethylmaleimide (3.5 mm; 18,200 cpm mol\(^{-1}\)) and \(N\-[\text{C}^4]\)ethylmaleimide (2.5 mm; 6,300 cpm mol\(^{-1}\)). The samples were incubated for 30 min at 37 °C, the reaction quenched, the samples denatured with SDS, and dialyzed. Samples (30 μg) were run on 5% SDS-polyacrylamide gels, stained, and scanned. The gels were then sliced and the slices submitted to liquid scintillation.](Image)
chain and very little with the $\beta$ chain. This result agrees with that obtained for the alkylation of purified rabbit kidney enzyme with N-ethylmaleimide (23). The amount of a chain present was determined by quantitative amino acid analysis. These values and the counts per min were used to calculate the amount of N-ethylmaleimide incorporated, and gave 3.9 ± 0.2 (n = 3) and 3.8 ± 0.1 (n = 6) mol of ESC (mol $\alpha$)$^{-1}$ for supernatant enzyme and SDS-purified enzyme, respectively.2

**Kinetics of Incorporation of Radio-labeled N-Ethylmaleimide into (Na$^+$ + K$^+$)-ATPase**—A sample of SDS-purified enzyme, in the absence of ligands and presumably stabilized with 1 M guanidine, was incubated with either Na$^+$ or K$^+$-ATPase. The amount of incorporation over 30, 60, and 120 min was determined. The results given in Table III show that after 30 min, an average of 4.0 mol of ESC (mol $\alpha$)$^{-1}$ is found regardless of the preparation of enzyme, range of specific radioactivity, or absolute concentration of N-ethylmaleimide. This substantiates the reproducibility of the conformational state as well as the reliability of the specific radioactivity determinations. Simultaneously, enzyme activity had declined by 70% over this time period consistent with the results from the kinetic inactivations.

A more complicated alternative to the earlier conclusion that only one sulfhydryl is involved in the inactivation of the enzyme would be that a rate-limiting reaction with one sulfhydryl group could be followed by the rapid reaction of several others, some or all of which caused partial or complete activity loss. The only additional incorporation of N-ethylmaleimide, however, during the next 90 min, 0.2 to 0.5 mol of ESC (mol $\alpha$)$^{-1}$, appears to correlate fairly closely with the loss of the remaining 20 to 30% of the activity (Table III). This indicates that 3 to 3.5 sulfhydryl groups have become rapidly and stoichiometrically alkylated by 30 min and do not react further, while only one other, the one responsible for inactivation continues to react after 30 min. Certainly, the most likely conclusion at this time is that only one sulfhydryl participates in the inactivation of the enzyme.

It has been shown that, during the isolation of membranes, oxidation of sulfhydryl groups can occur. These can be regenerated with 2-mercaptoethanol leading to increased reactivity with N-ethylmaleimide (49). To examine this possible source of confusion, (Na$^+$ + K$^+$)-ATPase, purified in the presence of 10 mM 2-mercaptoethanol from the initial kidney dissection until a final dialysis just prior to the labeling reaction, was included in these time course studies. No difference in the

| Table III | Kinetics of Incorporation of Radio-labeled N-Ethylmaleimide into (Na$^+$ + K$^+$)-ATPase |
|------------------------|-----------------------------------------------|
| Experiment | N-ethylmaleimide concentration (mol) | Specific radioactivity | Incorporation of N-ethylmaleimide (mol ESC) | Time of incubation (min) |
|-------------|-------------------------------|---------------------|-----------------------------|---------------------|
| Na$^+$ + K$^+$ | 2.5 | 6,300 | 3.9 (30%) | 0.0 (19%) | 4.2 (6%) |
| Na$^+$ + K$^+$ | 3.6 | 13,000 | 4.0 | 4.3 | 4.5 |
| Na$^+$ + K$^+$ | 2.6 | 3,100 | 4.0 | 4.3 | 4.5 |
| Na$^+$ + K$^+$ | 3.8 | 36,000 | 4.1 (35%) | 0.0 (21%) | 4.3 (9%) |

$^a$ SDS-purified enzyme prepared without 2-mercaptoethanol and isolated on separate occasions.

$^b$ SDS-purified enzyme prepared with 2-mercaptoethanol.

$^c$ N-[3H]Ethylmaleimide.

$^d$ N-[3H]Ethylmaleimide activity was also present at 3 to 4 mM and its specific radioactivity was determined immediately after mixing reagents together. The samples were incubated at 37 °C for 30 min, the reaction quenched, and large chain purified. Its specific radioactivity was determined by quantitative amino acid analysis and converted to moles of ESC for every 121,000 g of protein (mol of ESC (mol $\alpha$)$^{-1}$). The moles of "alkylated active site-SH" were calculated from inactivation values assuming that the inactivation was due to the alkylation of only one of the enzyme's sulfhydryl groups. The difference between total alkylation and active site alkylation is presented as "alkylated external-SH."
final level of incorporation was noted when these precautions were taken (Table III).

Since the ligand conditions used in these experiments produce the greatest levels of incorporation found in the studies now to be described, it follows that fluctuations in any of the variables examined in these initial experiments, namely N-ethylmaleimide concentration, method of preparation, or the presence of a reducing reagent, would not produce significant changes in the levels of N-ethylmaleimide incorporation under any of the ligand conditions examined in these experiments. Nevertheless, enzyme was routinely purified by the SDS procedure, including 10 mM 2-mercaptoethanol in all of the purification steps, and N-ethylmaleimide concentrations were kept as close to 3 to 4 mM as possible. Finally, three different methods for determining the protein concentrations of a chain were employed, and these yielded equivalent values.

**Incorporation of N-[3H]Ethylmaleimide by (Na+ + K+)-ATPase in the Presence of Ligands**—The quantitative procedures, justified in the preceding experiments, were applied to SDS-purified and nucleotide-free (Na+ + K+)-ATPase (Table IV). The enzyme was alkylated with N-[3H]ethylmaleimide for 30 min at 37°C under ligand conditions identical with those used in the kinetic inactivation experiments. Nevertheless, the extent of inactivation of each sample was measured. If it is assumed that the loss of enzyme activity is due to the alkylation of a single sulfhydryl group, then the molar fraction of that sulfhydryl which has been alkylated is equal to the fraction of the activity which has been lost. This amount, subtracted from the total incorporation, yields values for those reactive sulfhydryl groups, in addition to the activity-associated sulfhydryl, available for alkylation. These residues are referred to as external sulfhydryl groups. There are 2.8 ± 0.3 external sulfhydryl groups on the large chain of (Na+ + K+)-ATPase which react rapidly with N-ethylmaleimide when the enzyme is poised in the $E_1$ kinetic state; 1.9 ± 0.3, in the $E_2$-P state; 1.8 ± 0.3, in the $E_1$ state; and 1.9 ± 0.2, in the $E_1$ ~ P state. The results demonstrate that one additional sulfhydryl group, distinct from the one whose nucleophilicity is sensitive to levels of Na+ and K+ and whose alkylation inactivates the enzyme, is exposed upon stabilization of the enzyme in the $E_2$ kinetic state, and demonstrates a unique conformational form of the enzyme.

There was no change in the low level of incorporation into the β subunit regardless of the ligand conditions, indicating that any conformational change occurring in the α chain that would affect the β chain is not detected by sulfhydryl groups of the β subunit.

**DISCUSSION**

Four different sulfhydryl residues on the large chain of (Na+ + K+)-ATPase have been identified in the present experiments. The alkylation of one of them inactivates the enzyme. This one will be referred to as the activity-associated sulfhydryl. Two sulfhydryl groups are alkylated under all circumstances examined and will be referred to as the invariant sulfhydryl groups. Finally, one reacts only when the enzyme is alkylated while poised in the $E_2$ state and this one will be referred to as the $E_2$ sulfhydryl. These assignments serve to stress the fact that these residues are independent functionalities whose nucleophilicities respond to different changes in the state of the enzyme.

Although the results presented here are interpreted to identify four sulfhydryl groups of the α polypeptide that react with N-ethylmaleimide, only the isolation of individual modified peptides from N-ethylmaleimide-labeled α polypeptide would confirm the rhetorical equality drawn here between the incorporation stoichiometry and the actual presence of four reactive sulfhydryl groups. An analogy of this situation, however, has been encountered in the case of the reaction of N-ethylmaleimide with the sarcoplasmic reticulum Ca2+-ATPase, an enzyme whose single polypeptide closely resembles the α chain of the (Na+ + K+)-ATPase (50, 51). The analysis of peptides from Ca2+-ATPase (52) labeled with radioactive N-ethylmaleimide and digested with pepsin suggests that there is an equivalence between the amount of N-ethylmaleimide incorporated and the number of unique, reactive sulfhydryl groups in a protein closely related to (Na+ + K+)-ATPase.

The experiments described here utilize ligand combinations and concentrations which were recently demonstrated, in studies of other conformational probes, to stabilize exclusively a particular kinetic state of purified renal (Na+ + K+)-ATPase. It is under these conditions that the enzyme was reacted with an excess of either radiolabeled or nonradioactive N-ethylmaleimide. The pseudo-first order rate constants which describe the loss of enzymatic activity were measured and compared with the actual incorporation of radiolabeled N-ethylmaleimide under identical conditions. The results of these experiments demonstrate that the apparent nucleophilicity of the sulfhydryl residues of (Na+ + K+)-ATPase responds to two different signals. The rate of alkylation of the activity-associated sulfhydryl by N-ethylmaleimide is dependent on the presence of Na+ or K+ or the phosphorylation of the enzyme (Table I). If the contribution of this group is subtracted from the total incorporation, it can further be shown that the reaction of the $E_2$ sulfhydryl is controlled only by conformational state. Its alkylation, however, does not inactivate the enzyme.

The results of previous experiments which have followed ligand-associated structural changes of the α polypeptide by monitoring various reporters (Table V) have been interpreted in terms of a two-state isomerization of the enzyme between an $E_2$ and an $E_1$ conformation. It has been implied in these earlier reports that the $E_2$ and phosphorylated $E_2$-P kinetic states of the enzyme are conformationally equivalent and distinct from the $E_1$ and $E_1$-P states (7, 13, 15, 16, 53). The results presented here, however, illustrate clear changes when the $E_2$-P state becomes the $E_2$ state of the enzyme. In particular, the nucleophilicity of both the $E_2$ sulfhydryl (Table IV) and, less dramatically (Table I), the activity-associated sulfhydryl increase. Clearly, the transformation which produces a structural change in these regions of the catalytic subunit is not monitored by some of the other probes which have been employed. Furthermore, enzyme poised in the $E_2$ or $E_2$-P state displays the same unreactivity at the $E_2$ sulfhydryl as the $E_2$-P state even though structural differences between $E_2$-P and $E_1$ or $E_1$ ~ P at other locations of the protein have been observed by other methods (11, 13, 15, 16).

A close examination of Table V suggests that the evidence for only two exclusive conformational states has never been
very convincing. In particular, there are striking differences in ouabain affinity between \( E_0 \) and \( E_2 \) as well as \( E_1 \) and \( E_1-P \). In fact, ouabain may not even bind to any unphosphorylated form of the enzyme. While this value has been determined, the others represent lower limits.

In fact, ouabain may not even bind to any unphosphorylated form of the enzyme. While this value has been determined, the others represent lower limits.

The conclusion which can be reached as a rare comparison between \( E_1-P \) and any other form very convincingly. In particular, there are striking differences in fluorescence presence of ATP have been stated to be artifacts rarely have been made. The conclusion which can be reached is that there are at least four distinct conformations through which the enzyme passes during turnover, each displaying a different set of properties. These are \( E_1, E_1-P, E_2-P, \) and \( E_2 \). If this is the case, the question of when in the kinetic sequence the enzyme changes from an outward-facing form to an inward-facing form, and vice versa (60), remains an open one.

The possibility that even a fifth conformational state exists is raised by a comparison of the sulphydryl reactivity in the presence of saturating Na\(^+\) and ATP on the one hand and K\(^+\) and ATP on the other (Table I). Although structural differences between the enzyme in the presence of Na\(^+\) and K\(^+\) can be readily detected by changes in affinity for ATP (9-12, 37, 38), upon saturation with ATP, in the absence of Mg\(^{2+}\), the conformation adopted when either Na\(^+\) or K\(^+\) are present has been assigned as \( E_1 \) (13, 15). In addition, major structural differences between the \( E_2 \) state and that existing in the presence of saturating ATP and K\(^+\) have been observed in sulphydryl cross-linking studies (61, 62) and tryptic cleavage patterns (15, 17) which have also been interpreted as indicating that the enzyme is in the \( E_2 \) state under these circumstances. Yet the experimental results presented here, as well as in earlier studies (20), show that even when ATP is present at saturation, alkylation of the activity-associated sulphydryl is dramatically increased by the addition of K\(^+\) while that of the other accessible sulphydryl groups remains unchanged (Table IV). This effect of K\(^+\) presumably also results from a structural difference in the enzyme which alters the environment about this sulphydryl group, and which is not detected by the other methods. This additional conformation of the enzyme, stabilized by saturating concentrations of K\(^+\) and ATP, could possibly represent the contribution of a K\(^+\)-occluded state (38).

The differences in the nucleophilicity of the activity-associated sulphydryl in the presence of ATP and either K\(^+\) and Na\(^+\) also offers insight into the location of this sulphydryl on the catalytic subunit. Earlier reports have implied that the protection of enzymatic activity observed in the presence of ATP was due to the steric hindrance of a critical sulphydryl group located in the ATP binding site (22, 25). It has not been, however, decided whether the protection observed in the presence of ATP is a steric or an allosteric effect. There is now strong evidence which demonstrates that (Na\(^+\) + K\(^+\))-ATPase has a single, exclusive ATP binding site which is within the active site but which can be converted between high and low affinity forms depending on the presence of Na\(^+\) or K\(^+\), respectively (12, 63). In the case of the inhibition of enzymatic activity caused by N-ethylmaleimide in the presence of K\(^+\) and ATP (Table I), the ATP concentration utilized was saturating even considering the low affinity due to the simultaneous presence of K\(^+\). Therefore, the ATP binding site was as occupied as it was in the presence of Na\(^+\) or ATP alone. It follows that the protection observed when Na\(^+\) is exchanged for K\(^+\) (Table I) cannot be due to any steric protection, afforded by the binding of ATP, of a sulphydryl group located in the ATP binding site. An alternative location for the activity-associated sulphydryl could be near the cation binding site or in an area around the ATP binding site which responds allosterically to cation binding.

It is not clear which step of the kinetic cycle of the enzymatic reaction is blocked as a result of the alkylation of the activity-associated sulphydryl by N-ethylmaleimide. The most consistent observation concerning this question is that N-ethylmaleimide inhibits the ATPase activity more rapidly than the Na\(^+\)-dependent phosphorylation of the enzyme and that the properties of the phosphorylated intermediate change so that it can phosphorylate ADP in the presence of Na\(^+\) while becoming less sensitive to K\(^+\)-stimulated hydrolysis (8, 23, 24, 42, 55, 64, 65). These results have been interpreted as evidence for the existence of two phosphorylated states of the enzyme, \( E_1-P \) and \( E_2-P \), that are connected in the kinetic cycle by a conformational isomerization. It is this isomerization which is thought to be inhibited as a result of the alkylation of N-ethylmaleimide (8, 42, 65). Various degrees of inhibition of Na\(^+\)-dependent phosphorylation, however, have been observed in these studies, complicating this simple interpretation.
of the effects of N-ethylmaleimide (21, 23, 24, 55). When purified enzyme is incubated with N-ethylmaleimide in the presence of K+ and ATP, the modification which occurs produces only a slight inhibition of Na+-dependent phosphorylation (10% loss after 30 min) and substantial loss of ATPase activity (60% loss after 30 min) (24). In contrast to these results, it was observed that purified enzyme, at identical N-ethylmaleimide concentration and pH but in the absence of ligands, lost the ability to become phosphorylated to the same extent as the inhibition of ATPase activity (80% loss after 30 min) (21).

It is possible to explain these observations in terms of the results presented here in Tables I and IV. If only the E2 sulfhydryl is alkylated, the enzyme remains fully active (Table IV) and if only the activity-associated sulfhydryl is alkylated, as is the case when K+ and ATP are present, the transition between E1-P and E2-P is blocked, inactivating the enzyme (Table I) but not affecting Na+-dependent phosphorylation. If, and only if, however, both sulfhydryl groups are alkylated, as is the case when no ligands are present, Na+-dependent phosphorylation and overall turnover might be blocked coincidentally.

A coincident inhibition of ATPase activity and the ability of the enzyme to be phosphorylated is also produced by ATP analogues which alkylate sulfhydryl groups as well as the sulfhydryl oxidant DTNB (22, 26). Since the enzyme can be protected from both of these inhibitors by ATP, these results have been interpreted as evidence for the existence of a sulfhydryl group located within the ATP binding site. There is, in fact, a cysteine residue located in the sequence of the polypeptide very close to the aspartate residue that is, in fact, a cysteine residue located in the sequence of the enzyme. It should be noted that, while there are various electrophiles, it is not clear if the reaction of one of them with a certain sulfhydryl will produce the same effect as the reaction of N-ethylmaleimide with that same sulfhydryl.

Sorting out these various entanglements may be quite complicated.

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