*Biochimica et Biophysica Acta*, 422 (1976) 210—224


BBA 67682

# STUDIES ON ($Na^+$ + $K^+$)-ACTIVATED ATPase.

# XXXVIII. A 100 000 MOLECULAR WEIGHT PROTEIN AS THE LOW-ENERGY PHOSPHORYLATED INTERMEDIATE OF THE ENZYME

F.M.A.H. SCHUURMANS STEKHOVEN, M.P.E. VAN HEESWIJK, J.J.H.H.M. DE PONT and S.L. BONTING

*Department of Biochemistry, University of Nijmegen, Geert Grooteplein Noord 21, Nijmegen (The Netherlands)*

(Received June 12th, 1975)

## Summary

Phosphorylation of NaI-treated bovine brain cortex microsomes by inorganic phosphate in the presence of $Mg^{2+}$ and ouabain has been studied at 0°C (pH 7.4) and 20°C (pH 7.0). Nearly maximal (90%) and half-maximal phosphorylation are achieved at 20°C within 2 min with 50—155 and 5.6—17 $\mu$M $^{32}P_i$, respectively, and at 0°C within 75 s with 300—600 and 33—66 $\mu$M $^{32}P_i$, respectively. Maximal phosphorylation yields 146 pmol $^{32}P \cdot mg^{-1}$ protein. Without ouabain (20°C, pH 7.0) less than 25% of the incorporation observed in the presence of ouabain is reached.

Preincubation of the native microsomes with $Mg^{2+}$ and $K^+$, in order to decompose possibly present high-energy phosphoryl-bonds prior to ouabain treatment, does not affect the maximal phosphate incorporation. This indicates that the inorganic phosphate incorporation is not due to an exchange with high-energy phosphoryl-bonds, which might have been preserved in the microsomal preparations.

Phosphorylation of the native microsomes by ATP in the presence of $Mg^{2+}$ and $Na^+$ reaches 90 and 50% maximal levels within 15—30 s at 0°C and pH 7.4 at concentrations of [$\gamma$-$^{32}$P]ATP of 5—32 and 0.5—3.5 $\mu$M, respectively. The maximal phosphorylation level is 149 pmol $^{32}P \cdot mg^{-1}$ protein, equal to that of ouabain-treated microsomes phosphorylated by inorganic phosphate. Both inorganic phosphate and ATP phosphorylate one site per active enzyme subunit of 135 000 molecular weight.

From the equilibrium constants for the phosphorylation of ouabain-treated microsomes by inorganic phosphate at 0°C and 20°C standard free-energy changes of —5.4 and —6.8 kcal/mol, respectively, are calculated. These values yield a standard enthalpy change of 14 kcal/mol and an entropy change

of 70 cal/mol · °K. This characterizes the reaction as a process driven by an entropy change.

The intermediate formed by phosphorylation with $P_i$ has maximal stability at acidic pH, as is the case for the intermediate formed with ATP. Solubilization in sodium dodecyl sulfate stabilizes the phosphoryl-bond in the pH range of 4—7. The non-solubilized preparation has optimal stability at pH 2—4, the level of which is equal to that of detergent-solubilized intermediate.

Sodium dodecyl sulfate gel electrophoresis of the microsomes at pH 3, following incorporation of $^{32}P_i$ yields 11 protein bands, only one of which (mol. wt 100 000—106 000) carries the radioactive label. This protein has the same molecular weight as the protein, which is phosphorylated by ATP in the presence of $Mg^{2+}$ and $Na^+$.

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## Introduction

($Na^+$ + $K^+$)-stimulated ATPase (EC 3.6.1.3) is an enzyme located in the cell membrane and involved in the energy requiring process of $Na^+$ transport against a concentration gradient [1]. Its enzymatic mechanism probably involves two phosphorylated intermediates, a high-energy and a low-energy intermediate. The intermediate formed by the action of ATP in the presence of $Mg^{2+}$ and $Na^+$ is called the high-energy intermediate here because of its acylphosphate character (see next paragraph). It is thought to be transformed into a low-energy intermediate by means of a $K^+$-stimulated transphosphorylase step [2], which is then followed by hydrolysis. In the absence of $K^+$ and in the presence of ouabain as a stabilizing agent of the phosphorylated intermediates [3] phosphorylation with inorganic phosphate occurs, which reaction would involve reversal of the hydrolysis reaction and lead to a phosphorylated intermediate, which we provisionally assume to be of low-energy character.

The nature of the high-energy phosphorylated intermediate formed from ATP in the presence of $Mg^{2+}$ and $Na^+$ is fairly well established. It contains a β-aspartylphosphate bond [4] and is present in a polypeptide chain of molecular weight 84 000—103 000, as shown by sodium dodecyl sulfate gel electrophoresis of phosphorylated preparations of different origin [5—11]. The low-energy phosphorylated intermediate is less well established. Electrophoresis of radioactive fragments, resulting from pronase digestion of ($Na^+$ + $K^+$)ATPase preparations of different origin, yields identical patterns, whether the enzyme is phosphorylated by [γ-$^{32}$P]ATP or by $^{32}P_i$ [12,13]. The pronase fragment "$Pr_4$", which is a tripeptide of molecular weight of about 400, contains a serine or threonine residue adjacent to the high-energy β-aspartylphosphate group [4]. It has been suggested [2] that in the low-energy intermediate the phosphate would be attached to a serine group.

In this paper we report, in support of these findings, that phosphate incorporation under conditions yielding the low-energy intermediate takes place in a 100 000 molecular weight subunit, as determined via sodium dodecyl sulfate gel electrophoresis. According to its molecular weight, this subunit could be identical to the subunit carrying the high-energy phosphate bond after phosphorylation with ATP.

## Materials and Methods

*Preparation and assay of ($Na^+$ + $K^+$)ATPase*

NaI-treated bovine brain cortex microsomes are used as the enzyme preparation. Their preparation and specific ($Na^+$ + $K^+$)ATPase activity are presented elsewhere [14].

*Ouabain binding and phosphorylation by radioactive phosphate*

NaI-treated microsomes are preincubated for 30 min at 20°C at a protein concentration of 1 mg/ml in a buffer solution containing 50 mM imidazole/HCl, 5 mM $MgCl_2$ and 5 mM dithiothreitol (pH 7.0 or 7.4) in the presence or absence of 0.1 mM ouabain. Longer preincubation (up to 90 min) does not increase ouabain binding. The amount of ouabain bound, 350 pmol per mg protein, is the same when 1 mM inorganic phosphate is added to the preincubation medium. Phosphate incorporation is allowed to proceed for specified times at 0°C or at 20°C. The reaction is started by pipetting 1—2 ml aliquots of the preincubated suspension into test tubes containing 2—100 µl aqueous solution of 1—20 mM $^{32}$P-orthophosphoric acid (The Radiochemical Centre, Amersham, U.K.; specific radioactivity 200 Ci/mol). This order of addition results in faster mixing of the reaction components and hence more reproducible results than the reverse sequence.

The reaction is stopped by addition of 0.2 ml 50% (w/v) ice-cold trichloroacetic acid per ml of microsomal suspension, followed by 0.2 ml ice-cold 0.1 M sodium phosphate (pH 3.0). Equal aliquots of the preincubated microsomal suspension are used as zero-time controls; they are first denatured by addition of trichloroacetic acid and then added to the radioactive phosphate, followed by addition of 0.1 M sodium phosphate (pH 3.0). The suspensions are centrifuged at 0—4°C and 6000 × *g* for 15 min and the resulting pellets washed two times by resuspending them to the original volume in ice-cold 5% (w/v) trichloroacetic acid, containing 0.1 M $H_3PO_4$. They are then centrifuged as before.

Another method of interrupting the incorporation of radioactive inorganic phosphate has been used, which avoids the use of trichloroacetic acid. It is used in the determination of the pH stability pattern and preparation of phosphorylated material for gel electrophoresis described below. In this method the mixture is rapidly cooled in dry ice-acetone, centrifuged and the pellets washed two times with 0.1 M sodium phosphate (pH 3.0) at 0°C.

*Phosphorylation by radioactive ATP*

Phosphorylation of the NaI-treated microsomes by [$\gamma$-$^{32}$P]ATP at 0°C and pH 7.4 is carried out essentially as described by Post and Sen [15]. The microsomes are suspended in the buffer as used for $^{32}P_i$ incorporation without ouabain (pH 7.4), to which 100 mM NaCl is added. Samples of 1 ml suspension (1 mg/ml protein) are pipetted in test tubes containing 2—87 µl 0.06—1.2 mM [$\gamma$-$^{32}$P]ATP (sodium salt in 50% aqueous ethanol; The Radiochemical Centre, Amersham, U.K.; specific radioactivity 1260—1730 Ci/mol). This sequence of addition gives best reproducibility. No inhibitory effect of ethanol on the ($Na^+$ + $Mg^{2+}$ + ATP)-dependent phosphorylation level (steady state) is noticed in the

concentration range in our experiments (<0.5 M).

The reaction is stopped by addition of 0.2 ml 50% (w/v) ice-cold trichloroacetic acid, followed by 0.2 ml ice-cold 0.1 M non-radioactive ATP (pH 3.0). Centrifugation and washing is as described for the preparation following incorporation of $^{32}P_i$, except that the phosphoric acid is replaced by 0.1 M ATP (pH 3.0). As a control on the formation of side products, a phosphorylation medium is used in which 10 mM KCl replaces NaCl. The steady state of phosphorylation and dephosphorylation is calculated as the maximal difference between the $^{32}P$ incorporation in the $Na^+$ containing medium and that in the side product control.

*Effect of pH on the stability of the low-energy phosphorylated intermediate*

Batches of 20—25 ml ouabain-treated microsomes are mixed with 0.14—0.18 ml $^{32}P_i$, giving a final concentration of 56—78 $\mu$M $^{32}P_i$. After 2 min at 20°C and pH 7.0 the medium is cooled to near 0°C by immersion in acetone-dry ice, followed by centrifugation and washing as described in the second paragraph of this section. One batch is dissolved in 1% (w/v) sodium dodecyl sulfate and 1% (v/v) mercaptoethanol in 0.1 M sodium phosphate (pH 7.0), yielding a protein concentration of 5 mg/ml. Another batch is suspended in the same buffer without dodecyl sulfate. After 30 min at 37°C both mixtures are brought to 20°C and 0.5 ml aliquots are mixed with an equal volume of a 0.2 M sodium-phosphate buffer of the desired pH. The mixtures are incubated for 2½ h at 20°C. In some experiments a phosphate-free buffer containing 0.1 M each of sodium maleate, sodium citrate, Tris and imidazole has been used in order to determine whether any exchange of $^{32}P$ for non-radioactive phosphate occurs; no differences have been observed.

Next, 4 vols. of ethanol are added to the solubilized preparation and pH values below 3 are adjusted with 2 M NaOH to pH 3, to achieve precipitation in all cases. Both sets of preparations are centrifuged at room temperature (15 min, 10 000 × *g*) and washed two times at 0—4°C with 0.1 M sodium phosphate (pH 3.0) by resuspension and centrifugation as indicated. Final pellets are resuspended in 1 ml of the same buffer. 50-$\mu$l aliquots are taken for determination of protein, and the remainder is counted for $^{32}P$.

*Sodium dodecyl sulfate gel electrophoresis*

Sodium dodecyl sulfate gel electrophoresis at pH 3.0 is carried out according to Fairbanks and Avruch [16] in a climate chamber thermostated at 16—20°C. The microsomal material is solubilized with dodecyl sulfate as described in the previous paragraph. After 30 min at 37°C the pH is adjusted to 3.0 by addition of 1 M HCl, followed by addition of 0.3 ml tracking dye solution (0.1 mg/ml Pyronin Y and 0.24 g/ml sucrose) per ml of solubilized microsomes. A 0.1 ml aliquot of this mixture, containing about 0.4 mg of protein, is placed on each gel.

Calibration proteins are *Escherichia coli* $\beta$-galactosidase, chicken egg albumin, rabbit muscle pyruvate kinase, bovine serum albumin and rabbit muscle phosphorylase *a* (all from Boehringer, Mannheim GmbH, Germany) and bovine serum albumin (Behringwerke AG, Marburg Lahn, Germany). All, except $\beta$-galactosidase, show single bands on electrophoresis and are therefore solubi-

lized and subjected to electrophoresis together on one gel. As molecular weights of the calibration proteins the values reported by Weber and Osborn [17] are used. Since β-galactosidase shows two major bands and some fainter ones, it has been dissolved and subjected to electrophoresis separately but simultaneously, the slower moving of the two major bands being taken as the 130 000 molecular weight protein.

After termination of electrophoresis the position of the tracking dye is marked on each gel as the point of mobility 1.0. Gels, which are used for tracing radioactive phosphate, are cut in 2 mm slices [18], and each slice is weighed in a counting vial. The gels are stained and destained according to Fairbanks et al. [19] and scanned spectrophotometrically at 550 nm on a linear absorbance scale.

*Determination of protein*

Protein is determined according to Hess and Lewin, method C [20]. Unless otherwise indicated, phosphorylated microsomes and their controls after the final wash are suspended in the original volume of distilled water and 0.1—0.15 ml is used for determination of protein.

*Determination of radioactivity*

The suspension is mixed with 10 ml Aquasol (New England Nuclear). Slices from radioactive gels are extracted overnight at 50°C in 1 ml Protosol (New England Nuclear) before addition of 10 ml Aquasol. Radioactivity is counted in a liquid scintillation analyzer programmed for $^{32}P$. Known amounts of $^{32}P_i$ or [$\gamma$-$^{32}P$] ATP in the same scintillation media are counted concurrently in order to allow conversion of cpm to pmol. All counts are corrected for back-ground.

## Results

*Formation of the phosphoenzyme with inorganic phosphate*

Optimal $^{32}P_i$ incorporation in the ouabain-treated microsomal preparation is obtained at pH 7.4 and 37°C, confirming the results of Lindenmayer et al. [21]. In view of the rapid decrease in incorporation above the optimal pH and temperature, the experiments have been carried out at pH 7.0 and 20°C. Fig. 1 shows that under those conditions, incorporation is complete after 2 min of incubation. Without ouabain treatment $^{32}P_i$ incorporation is less than 5% of that after ouabain treatment (Fig. 1). This emphasizes, in agreement with other authors [13,21—24], the need for ouabian treatment in studies of $^{32}P_i$ incorporation.

The optimal $^{32}P_i$ concentration for incorporation has also been determined. Fig. 2a shows that at 20 μM $^{32}P_i$ incorporation is still suboptimal (86%) after 2 min incubation. In another experiment with a different enzyme preparation, higher concentrations of $^{32}P_i$ (25—100 μM), but in the presence of ouabain, give a further increase up to the maximal phosphorylation capacity of the membranes. The phosphorylation curves obey the simple Michaelis-Menten for-

mulation [25] for the equilibrium

$$\underset{(e_0 - y)}{E} + \underset{(x)}{P_i} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(x)}{E\text{-}P} + \underset{(y)}{H_2O} \qquad (1)$$

where E represents the ouabain-Na-K ATPase complex, $e_0$ the initial concentration of this non-phosphorylated complex, E-P its phosphorylation product, $y$ the concentration of E-P, $x$ the concentration of $^{32}P_i$ and $k_1$ and $k_2$ the rate constants for phosphorylation and dephosphorylation, respectively. The relation between $x$ and $y$ is then given by:

$$\frac{1}{y} = \frac{k_2}{k_1} \cdot \frac{1}{e_0 x} + \frac{1}{e_0} \qquad (2)$$

It is plotted for the experimental data of Fig. 2a in Fig. 2b ($y$ in cpm · $mg^{-1}$ protein, $x$ in $\mu$M). Intersection with the abscissa is at $-k_1/k_2$, yielding a $K_m$ ($=k_2/k_1$) value of 5.6 $\mu$M. The intersection with the ordinate is at $1/e_0$ yielding a maximal incorporation of $^{32}P_i$ of 35 000 cpm · $mg^{-1}$ protein (150 pmol $^{32}P$ · $mg^{-1}$ protein).

In subsequent experiments (Figs. 4 and 5) higher $^{32}P_i$ concentrations (56—385 $\mu$M) have been used, which according to extrapolation of the regression line in Fig. 2b should provide 91—99% maximal phosphorylation. A similar regression line (not shown in Fig. 2b), calculated from data for the non-

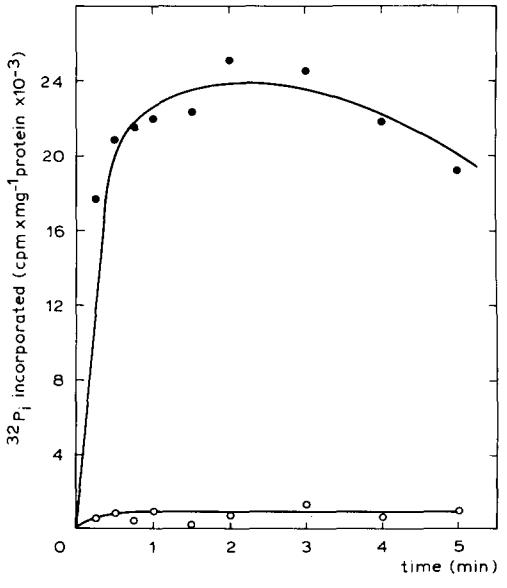



**Fig. 1. Phosphate incorporation into NaI-treated bovine brain microsomes as a function of time. Ouabain-treated microsomes ●———●, non-ouabain-treated microsomes ○———○. The concentration of $^{32}P_i$ is 20 $\mu$M. The reaction was carried out at 20°C and pH 7.0, and stopped at the indicated times by addition of trichloroacetic acid as described under Materials and Methods.**

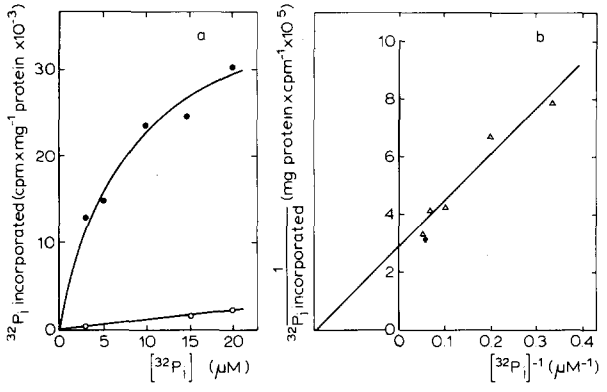



Fig. 2. Phosphate incorporation into NaI-treated brain microsomes as a function of the radioactive phosphate concentration. (a) Ouabain-treated microsomes ●———●, non-ouabain-treated microsomes ○———○. Reaction time is 2 min; further experimental conditions are as in Fig. 1. (b) Reciprocal $^{32}P_i$ incorporation versus reciprocal $^{32}P_i$ concentration. The line through the points is calculated according to the least squares method using data for ouabain-treated microsomes from upper curve in a.

ouabain-treated samples (bottom line in Fig. 2a), results in 13—22% of the $^{32}P_i$ incorporation computed for the ouabain-treated microsomes in this phosphate concentration range. The maximum at infinite $x$ is 25%.

The incorporation of $^{32}P_i$ might be caused by exchange with high-energy phosphoryl bonds still present in the microsomes, thus yielding bonds of the same energy level. This possibility has been investigated by preincubating the preparation with $Mg^{2+}$ and $K^+$, which should give a rapid decay of any phosphorylated intermediates already present in the preparation [26]. Preincubation is for 10 min at 20°C and pH 7.0 in the presence of 5 mM $MgCl_2$ and 10 mM KCl, which is followed by washing to remove $K^+$ and by the usual ouabain-

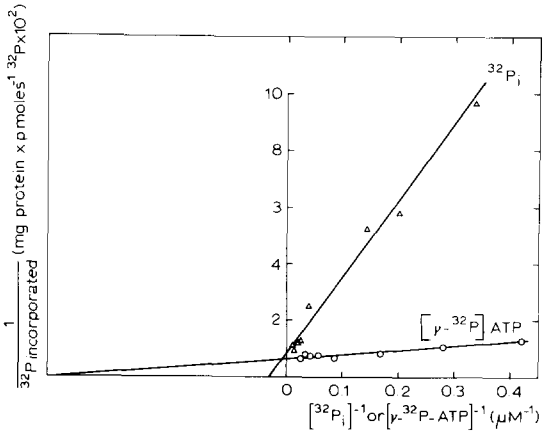



Fig. 3. Double reciprocal plot of steady-state levels of phosphorylation by [$\gamma$-$^{32}$P]ATP or $^{32}P_i$ versus substrate concentrations. Phosphorylation by inorganic phosphate is determined over 75 s at 0°C and pH 7.4 on NaI-treated bovine brain microsomes, pretreated with ouabain. Phosphorylation by [$\gamma$-$^{32}$P]ATP is similarly determined over 30 s on non-ouabain-treated microsomes. Lines through the points are calculated according to the least squares method.

treatment. $^{32}P_i$ incorporation for 2 min at pH 7.0 and 20°C yields a maximum of 133 pmol $^{32}P \cdot mg^{-1}$ protein, as compared to 139 pmol $^{32}P \cdot mg^{-1}$ protein for a control preincubated without $K^+$. This result indicates that phosphorylation by inorganic phosphate is not caused by exchange with pre-existing high-energy phosphoryl bonds.

*Relationship to the phosphoenzyme formed by ATP*

The number of phosphorylation sites for $P_i$ and for ATP has been compared. The results suggest a 1 : 1 ratio for the high- and low-energy phosphorylated intermediates. Fig. 3 shows a double reciprocal plot of the steady state level of phosphorylation by [$\gamma$-$^{32}P$] ATP versus its concentration and a similar plot for phosphorylation by $^{32}P_i$ of ouabain-treated microsomes. A temperature of 0°C has been used in both cases in order to prevent early exhaustion of ATP at the low concentrations and to extend the steady-state period between phosphorylation and hydrolysis of the phosphorylated intermediate to several seconds. A pH of 7.4 was chosen for these experiments, since pH values between 7.3 and 7.6 have been used commonly by other investigators [27—29].

This steady state of ($Na^+ + Mg^{2+}$)-dependent phosphorylation by ATP can be described by:

$$\underset{(e_0 - y)}{E} + \underset{(x)}{ATP} \xrightarrow[Na^+ + Mg^{2+}]{k_1} \underset{(y)}{E \sim P} + ADP \xrightarrow[H_2O]{k_3} E + P_i \qquad (3)$$

Reversal of the phosphorylation step will be negligible at the high $Mg^{2+}$ concentration (5 mM) used in these experiments [30]. In this equation, E represents the native enzyme, $e_0$ its initial concentration, E~P its phosphorylation product, $y$ the concentration of E~P, $x$ the ATP concentration and $k_1$ and $k_3$ the rate constants for formation and hydrolysis of E~P. In the steady state the relation between $x$ and $y$ is given by the equation [25]:

$$\frac{1}{y} = \frac{k_3}{k_1} \cdot \frac{1}{e_0 x} + \frac{1}{e_0} \qquad (4)$$

This equation is similar to equation (2) for the incorporation of $^{32}P_i$, but now the regression line through the experimental points of $1/y$ versus $1/x$ intersects with the abscissa at $-k_1/k_3$ and the slope is $k_3/k_1 e_0$. From the intercepts with the ordinate ($1/y$ expressed in $pmol^{-1}$ $^{32}P \cdot$ mg protein, Fig. 3) the maximal phosphorylation capacities for $P_i$ and ATP can be calculated. In three experiments the ratio for the phosphorylation capacity for $P_i$ to that for ATP is found to be 1.00 (S.E. 0.12; Table I), indicating that there is a 1 : 1 ratio between the number of sites that can be phosphorylated by $P_i$ and those that can be phosphorylated by ATP in the presence of $Na^+$ and $Mg^{2+}$.

The linear relationship between the reciprocals of $y$ and $x$, observed in Figs. 2b and 3, indicates that the sites for each type of phosphorylation are independent of each other (no cooperative or anti-cooperative effects) and are of the same chemical nature [31].

TABLE I

PHOSPHORYLATION OF NaI-TREATED MICROSOMES BY $^{32}P_i$ OR [$\gamma$-$^{32}$P]ATP

Maximal levels ($e_0$), ratios of kinetic constants for formation ($k_1$) and hydrolysis ($k_2$ or $k_3$), ratios of the maximal extent of formation ($e_{0P_i}/e_{0ATP}$), and standard free-energy change of formation from $P_i$ ($\Delta G_0$) are given. The values for $e_0$, $k_1/k_2$ and $k_1/k_3$ are derived from the intercepts on ordinate and abscissa of regression lines fitting the experimental points (cf. Fig. 2b and 3). Phosphorylation of ouabain-treated microsomes by $P_i$ (3—100 $\mu$M) is for 2 min at 20°C and pH 7.0 and for 75 s at 0°C and pH 7.4; phosphorylation of non-ouabain treated microsomes by ATP (2.5—42 $\mu$M) at 0°C and pH 7.4 is for 15—30 s. Further procedure is described under Materials and Methods.

| | $e_0$ $\left(\frac{\text{pmol } ^{32}P}{\text{mg protein}}\right)$ | $k_1/k_2$ ($M^{-1}$) | $\Delta G_0$* (kcal/mol) | $e_0$ $\left(\frac{\text{pmol } ^{32}P}{\text{mg protein}}\right)$ | $k_1/k_3$ ($M^{-1}$) | $\frac{e_{0P_i}}{e_{0ATP}}$ |
|---|---|---|---|---|---|---|
| 20°C, pH 7.0, substrate $^{32}P_i$ | | | | | | |
| Expt. 1 | 150 | $1.8 \cdot 10^5$ | —7.1 | | | |
| Expt. 2 | 139 | $0.6 \cdot 10^5$ | —6.4 | | | |
| Average | 145 | $1.2 \cdot 10^5$ | —6.8 | | | |
| 0°C, pH 7.4, substrate (a) $^{32}P_i$ | | | | (b)[$\gamma$-$^{32}$P]ATP | | |
| Expt. 1 | 121 | $0.30 \cdot 10^5$ | —5.6 | 162 | $4.0 \cdot 10^5$ | 0.75 |
| Expt. 2 | 161 | $0.26 \cdot 10^5$ | —5.5 | 143 | $2.8 \cdot 10^5$ | 1.12 |
| Expt. 3 | 161 | $0.15 \cdot 10^5$ | —5.2 | 144 | $18 \cdot 10^5$ | 1.12 |
| Average | 148 | $0.24 \cdot 10^5$ | —5.4 | 150 | $8.3 \cdot 10^5$ | 1.00 |

* Calculated from $k_1/k_2$ by means of the formula $\Delta G_0 = -2.3\ RT \log k_1/k_2$.

The average maximal phosphorylation capacity of the NaI-treated microsomes is 148 pmol $^{32}P \cdot mg^{-1}$ protein (Table I) and their average specific ($Na^+ + K^+$)-ATPase activity is 0.5 μmol ATP hydrolyzed per mg protein per min [14]. Assuming an average specific activity of 25 $\mu mol \cdot mg^{-1}$ protein $\cdot min^{-1}$ for a purified ($Na^+ + K^+$)-ATPase preparation [32], this would mean that (1) the ($Na^+ + K^+$)-ATPase content of the NaI-treated microsomes is about 2%, and (2) in a purified preparation 1 mol $^{32}P$ would bind to a 135 000 molecular weight subunit. The latter value agrees with that found (137 000) by Jørgensen [33] for the active enzyme subunit of purified ($Na^+ + K^+$)-ATPase from rabbit kidney outer medulla.

*Thermodynamic data for the formation of the phosphoenzyme*

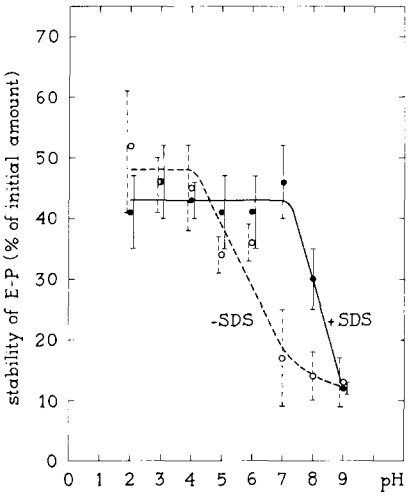
The intercept with the abscissa in the left hand quadrant for $P_i$ incorporation (Figs. 2b and 3) gives the equilibrium constant $k_1/k_2$ for this reaction. This permits calculation of the standard free energy change at 0° and 20°C by means of the relation:

$$\Delta G_0 = -2.3\ RT \log k_1/k_2$$

The resulting values of −6.8 kcal $\cdot mol^{-1}$ at 20°C and pH 7.0 and of −5.4 kcal $\cdot mol^{-1}$ at 0°C and pH 7.4 (Table I) indicate that the phosphorylation reaction is exergonic. Disregarding the small pH difference at the two temperatures and phase transitions that may occur between 0° and 20°C [34], values of 14±4 kcal $\cdot mol^{-1}$ and 70±20 cal $\cdot mol^{-1} \cdot °K^{-1}$ for the standard enthalpy and entropy change, respectively, can be calculated for this phosphorylation reaction. These values for $\Delta H_0$ and $\Delta S_0$ indicate that the entropy change is the driving force for the reaction, as previously suggested by Dahl and Hokin [32]. The values of the intercepts with the abscissa ($k_1/k_3$) for steady state phosphorylation by ATP are 13—120 times as large as those found for phosphorylation by $P_i$ (Table I). The free-energy change cannot be calculated from the value of $k_1/k_3$, since $k_3$ refers to the release of free phosphate rather than to the reverse phosphorylation reaction.

*Effect of pH on the stability of the phosphoenzyme*

The effect of the pH on the stability of the phosphate bond has been determined in the following way. Ouabain-treated microsomes are phosphorylated by inorganic phosphate, and are then solubilized in sodium dodecyl sulfate and are finally incubated for 2.5 h at 20°C and at the pH indicated on the abscissa in Fig. 4. For comparison, the pH stability pattern of the phosphorylated preparation which is not solubilized, but has otherwise undergone the same treatment, is shown in this figure. The stability is expressed as percent of the $^{32}P$ bound to the preparation prior to solubilization. Optimal stabilization for the non-solubilized preparation is obtained between pH 2 and 4, followed by a substantial decrease in stability at higher pH. Solubilization in sodium dodecyl sulfate does not significantly affect the level of optimal stabilization, but the start of the decrease in stability is shifted from pH 4 to pH 7. This difference between the two curves suggests that detergent-induced denaturation confers a greater stability on the phosphate bond. Similar effects have been



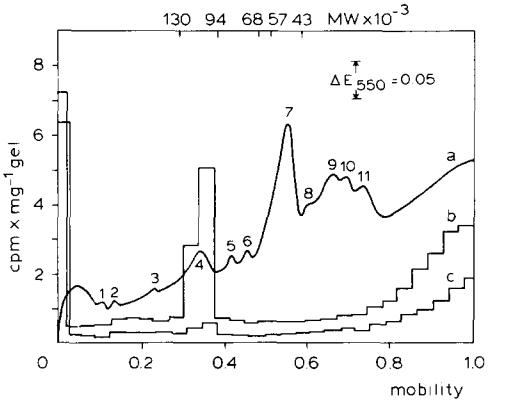




Fig. 4. The pH-stability pattern of the phosphorylated intermediate E-P. NaI-treated bovine brain microsomes, pretreated with ouabain, after incorporation of inorganic phosphate o———o. The effect of solubilization in dodecyl sulfate (SDS) is also shown ●———●. Averages plus standard errors for three experiments are plotted in this figure. The stability of the phosphoryl-bond is expressed on the left hand ordinate as the relative amount of radioactive phosphate remaining protein-bound. The protein-bound $^{32}$P-level prior to solubilization of the membranes is taken as 100% (133 pmol $^{32}$P · mg$^{-1}$ protein).

Fig. 5. Sodium dodecyl sulfate gel electrophoregram of NaI- and ouabain-treated bovine brain cortex microsomes after $^{32}P_i$ incorporation in the presence of 385 $\mu$M $^{32}P_i$ (2 min at 20°C and pH 7.0). Electrophoresis is carried out at 16°C. Radioactivity is expressed as cpm · mg$^{-1}$ gel on the left ordinate. (a) Absorbance scan at 550 nm of a reference gel stained for protein; (b) radioactivity pattern of an unstained gel containing microsomal proteins following ouabain treatment and $^{32}P_i$ incorporation; (c) same as (b) but omitting ouabain treatment. The positions and molecular weights of proteins used for molecular weight calibration are indicated at the top of the diagram.

observed for the enzyme phosphorylated by ATP in the presence of $Na^+$ and $Mg^{2+}$ [10].

*Identification of the phosphoenzyme by gel electrophoresis*

For gel electrophoresis the preparation is solubilized in the detergent at pH 7.0 for 30 min, since lower pH values result in incomplete solubilization or require extended solubilization times. Once solubilization has been achieved, the pH is brought within the range of maximal stabilization (pH 3) and electrophoresis is carried out for 3 h at the same pH at or below 20°C, which allows the tracking dye to travel 56—60 mm.

Fig. 5 (curve a) shows the protein pattern of the NaI- and ouabain-treated bovine brain cortex microsomes after $^{32}P_i$ incorporation and following sodium dodecyl sulfate gel electrophoresis. Reaction of the microsomes with ouabain and $^{32}P_i$ does not change the pattern and the mobilities of the protein bands. The material after NaI treatment is still rather crude, as shown by the many (11) bands displayed in the pattern. Two prominent bands are a 100 000—106 000 molecular weight protein (peak no. 4; mobility 0.350—0.365) and a 48 000—51 500 molecular weight protein (peak no. 7; mobility 0.551—0.569), which are present also in more purified bovine brain cortex ($Na^+$ + $K^+$)-ATPase

preparations [5]. Only the former band is phosphorylated by $^{32}P_i$ in the presence of ouabain (Fig. 5, pattern b), but not in its absence (Fig. 5, pattern c). Its molecular weight is equal to that of the protein phosphorylated by ATP in the presence of $Mg^{2+}$ and $Na^+$ (94 000—102 000) for various brain preparations [5,7,10,35].

The yield of radioactivity in peak 4 is 10—14% at an electrophoresis temperature of 16—20°C. This recovery is intermediate between yields in radioactivity at this peak following sodium dodecyl sulfate gel electrophoresis at pH 6—7.1 and 20°C (4—8%) [5,35] and at pH 8.3 and 0—4°C (30%) [8] of material phosphorylated by [$\gamma$-$^{32}$P] ATP. We have used the higher temperature range, since electrophoresis at 0—4°C gives precipitation of dodecyl sulfate under our conditions (pH 3). In the absence of ouabain (Fig. 5, pattern c) the radioactivity in peak 4 is 13% of that in the presence of ouabain (Fig. 5, pattern b), which is in reasonable agreement with an expected value of 22% computed by extrapolation from data of Fig. 2b. The radioactivity at the top of the gel is probably due to poorly penetrating protein. At an electrophoresis temperature of 16°C it amounts to 2—7% of the radioactivity originally incorporated into the microsomes. This fraction decreases to 0.2% when electrophoresis takes place at 20°C. Peak 4 is followed by a gradual increase of radioactivity at a mobility of 0.750 or higher. This activity is not bound to protein, since no Coomassie brilliant blue stained bands can be detected beyond peak 11 (Fig. 5, curve a). This gradual increase in radioactivity at the lower end of the gel is not detected in runs at 20°C and is apparently due to $^{32}P_i$, which is released and leaves the gel before the end of the run at the higher electrophoresis temperature. Similar findings have been reported by others after phosphorylation of microsomal material by [$\gamma$-$^{32}$P] ATP in the presence of $Mg^{2+}$ and $Na^+$ [5,6,8].

## Discussion

*Identity of phosphoproteins*

We have shown that ouabain- and $Mg^{2+}$-dependent phosphorylation of bovine brain cortex ($Na^+$ + $K^+$)ATPase by inorganic phosphate takes place in a protein subunit with the same molecular weight (100 000—106 000) as that phosphorylated by ATP in the presence of $Mg^{2+}$ and $Na^+$ (molecular weight 94 000—102 000) [5,10]. A similar finding has been reported by Collins and Albers [7] for eel electroplax microsomes in less detail and omitting incorporation of $^{32}P_i$ in the absence of ouabain. An early finding of Chignell and Titus [36] for rat kidney microsomes also suggests that a protein of the same molecular weight is phosphorylated in both systems, but their method of gel electrophoresis in phenol/acetic acid/urea gives poorer resolution than that in sodium dodecyl sulfate [37].

Evidence that the same polypeptide subunit of ($Na^+$ + $K^+$)-ATPase is involved in the two types of phosphorylation can be summarized as follows. The 100 000 molecular weight protein is the only subunit which is phosphorylated by ATP [5—10] under formation of the high-energy phosphorylated intermediate [4,38]. Pronase or pepsin digests of crude microsomes after phosphorylation by [$\gamma$-$^{32}$P] ATP or $^{32}P_i$ give identical radioelectrophoregrams [12,13].

Yet these findings do not completely rule out the possibility that different protein subunits are involved, which have the same charge:mass ratio and yield the same radioactive peptides upon enzymatic proteolysis. This possibility is not as unlikely as it might seem. For instance, even distantly related enzymes like $(Na^+ + K^+)$-ATPase and $Ca^{2+}$-ATPase yield the same limit phosphopeptides $Pr_4$ (molecular weight 400) or $P_6$ (molecular weight 2000), respectively, following phosphorylation from ATP and proteolysis by pronase or pepsin [39]. Only fragments larger than the limit peptic phosphopeptide ($P_6$) derived from both enzymes show different electrophoretic behaviour [39]. These results suggest that phosphorylation of $(Na^+ + K^+)$-ATPase by ATP or $P_i$ could occur on different polypeptides of the same molecular weight and covering the same active centre. This possibility would be in agreement with the suggestion of Robinson [40] that different steps in the $(Na^+ + K^+)$-ATPase reaction sequence are catalyzed by different subunits of the enzyme.

*The phosphoprotein as a reaction intermediate of $(Na^+ + K^+)$-ATPase*

Involvement of a 100 000 molecular weight phosphoprotein in the reaction mechanism of $(Na^+ + K^+)$-ATPase is well established. It is formed in the $(Na^+ + Mg^{2+})$-dependent phosphorylation by ATP and loses phosphate upon addition of $K^+$ [5]. The phosphorylated intermediate is stabilized by ouabain against $K^+$-activated hydrolysis [3,12], which substance is a specific inhibitor of $(Na^+ + K^+)$-ATPase activity [1]. Ouabain binds to a 100 000 molecular weight subunit of this enzyme [41,42] and its binding is, as shown in this paper, a prerequisite for phosphorylation of such a subunit by inorganic phosphate.

$K^+$-stimulated phosphatase activity is thought to represent one of the last steps in the reaction sequence of $(Na^+ + K^+)$-ATPase, since it is enriched to the same extent during purification of the latter enzyme [5]. Also, *p*-nitrophenyl phosphate hydrolysis by the enzyme passes through a phosphorylated intermediate, which is stabilized by ouabain against the action of $K^+$ and which can exchange phosphate with $P_i$ [43].

These data provide sufficient evidence to assume that the 100 000 molecular weight subunits, whether phosphorylated by ATP or $P_i$, represent true reaction intermediates. In line with current ideas about a transphosphorylation step in the reaction mechanism of $(Na^+ + K^+)$-ATPase [2], both intermediates appear to occur in a 1 : 1 ratio, as has previously been suggested for electric eel electroplax [24].

*The energy of the phosphate-enzyme bond*

The incorporation of inorganic phosphate into the enzyme is strongly exergonic with a standard free energy change of —6.8 kcal/mol at 20°C and pH 7.0. It is not yet established whether the formed product contains a phosphate ester or an aspartylphosphate. For the formation of water soluble phosphate esters, the standard free energy of formation is about +4 kcal/mol (ranging from +2.3 to +5 kcal/mol) [44] and for the formation of β-aspartyl phosphate from free aspartic acid and $P_i$ it even amounts to +11.5 kcal [44]. This indicates that the formed phosphorylated compound has a standard free energy of formation, which is 10.8 kcal/mol lower than for water soluble phosphate

esters and 18.3 kcal/mol lower than for aspartylphosphate. In either case the formation of the intermediate will require a substantial amount of additional energy. This additional energy is most likely supplied by a conformational change in the ($Na^+$ + $K^+$)-ATPase complex, caused by the reaction with ouabain. This suggestion is supported by the high entropy change for the phosphorylation reaction (70 cal · $mol^{-1}$ · $°K^{-1}$), calculated by us.

The behaviour of the low-energy phosphorylated intermediate towards hydroxylamine [12,43] and towards pH change [13] is strikingly similar to that of the high-energy phosphorylated intermediate, which would seem to favour an acyl-bound phosphate in both cases. However, Robinson [45] finds the $K^+$ stimulated phosphate incorporation from *p*-nitrophenyl phosphate to be largely insensitive to hydroxylamine. Furthermore, the pH-stability pattern is dependent on the influence of neighbouring groups near the active centre rather than on the stability of the phosphoryl-bond per se [46]. So this pattern may not distinguish between low- and high-energy phosphate groups, when they are located in the same active centre and undergo the same destabilizing influence by their amino acid environment.

It is obvious from the above consideration that the nature of the enzyme-phosphate bond formed by phosphorylation with inorganic phosphate is still undetermined. It has become fairly certain, however, that this phosphate incorporation is coupled to an exergonic process, which is driven by an entropy change. A similar process is also involved in the incorporation of inorganic phosphate into $Ca^{2+}$-ATPase of sarcoplasmatic reticulum [47], and its standard enthalpy (15.9 kcal/mol) and entropy change (50.2 cal/mol · °K) are strikingly similar to the values found in our system.

## Acknowledgements

Expert technical assistance was provided by Mr. H.Th.B. van Moerkerk. This study was subsidized by a grant from the Netherlands Organization for Basic Research (Z.W.O.) through the Netherlands Foundation for Chemical Research (S.O.N.).

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