The Steady State Level of Phosphorylated Intermediate in Relation to the Two Sodium-dependent Adenosine Triphosphatases of Calf Brain Microsomes*

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SUMMARY

During ATP hydrolysis, microsomal preparations form an acid-stable protein-phosphate complex. Since there is evidence that two different independent enzymatic sites catalyze the hydrolysis of ATP in calf brain microsomes, it was reasoned that either one or both of these might contribute to the formation of this complex. To determine the actual relationships, the phosphorylation reaction was compared with hydrolysis at each of the ATPase sites—at different levels of Na⁺, K⁺, and ATP and at different temperatures. The kinetic analysis indicates that it is hydrolysis at Site I, the Na⁺-K⁺ ATPase, which correlates with the steady state level of protein-phosphate complex. The results add support to the hypothesis that this complex is an intermediate in the hydrolysis of ATP by the Na⁺-K⁺ ATPase—the site that takes part in Na⁺ and K⁺ transport across biological membranes. The second ATPase, Site II, is a Na⁺-ATPase inhibited by K⁺; the kinetic analysis indicates that this site does not contribute substantially to the steady state level of acid-stable phosphorylated intermediate. It is suggested that Site II may be related to a sodium pump such as the one in red blood cells described by J. F. Hoffman and F. M. Kregnow (Ann. N. Y. Acad. Sci., 137, 566 (1966)).

In an earlier paper, we presented evidence that two different ouabain-sensitive enzymatic sites independently catalyze the hydrolysis of ATP in calf brain microsomes (2); similarly, Czerwinski, Gitelman, and Welt have presented evidence for these two different sites in rat erythrocytes (3). One of these (Site I) is the Na⁺-K⁺ ATPase considered to be part of the mechanism for Na⁺ and K⁺ transport across biological membranes (4, 5). The other (Site II) is postulated to be a Na⁺-ATPase inhibited by K⁺. This second site may play a role in one of the many transport systems that require Na⁺ but not K⁺ (6, 7).

In the presence of Mg²⁺, Na⁺, and ATP, microsomal preparations from a variety of tissues form an acid-stable protein-phosphate complex that breaks down in the presence of K⁺. Considerable evidence suggests that this complex is an intermediate in the hydrolysis of ATP by the Na⁺-K⁺ ATPase (Site I) (8-10). However, with the recognition of a second Na⁺-dependent ATPase (Site II) in microsomal preparations, the question arose to what extent this Na⁺-ATPase contributes to the phosphorylation reaction. To answer this, we compared hydrolysis at each of the sites with the phosphorylation reaction, as functions of Na⁺, K⁺, ATP, and temperature. The results support the conclusion that it is Site I, the Na⁺-K⁺ ATPase, which forms the protein-phosphate intermediate; the kinetic analysis shows how the formation and breakdown of this intermediate relates to steps in the mechanism of hydrolysis at this site.

MATERIALS AND METHODS

Preparation of Calf Brain Microsomes

Calf brain microsomes were prepared by a modification of the method of Schoner et al. (11) as previously described (3).

Preparation of ATP³²P

ATP³²P was prepared by a modification of the method of Glynn and Chappell (12) as previously described (3).

Measurement of Acid-stable Enzyme-³²P Complex

The procedure for showing ³²P labeling of protein from ATP³²P was a modification of the method described by Post, Sen, and Rosenthal (13). The amount of phosphate complex was determined by measuring the specific activity of acid-precipitated protein after incubation in the appropriate reaction medium. In a typical experiment, 3.0 ml of enzyme suspension (3.5 mg of protein per ml) were added to 100 ml of a cold solution having the following composition: 100 mM NaCl, 5 mM MgCl₂, 100 mM imidazole, pH 7.3. For a single experiment, 5.0 ml of this solution was used with 0.05 ml of water or 0.05 ml of 1 M KCl. The mixture was brought to 37° by incubation for 15 min...
in a thermostatic water bath. The reaction was started with 0.5 ml of ATP and stopped 15 sec later with 20 ml of fresh, cold 0.4 M trichloracetic acid containing 0.6 mM ATP. This mixture was centrifuged. The supernatant was discarded, and the precipitate was washed twice with 20 ml of fresh, cold 0.4 M trichloracetic acid. All centrifugations were at 10,000 \( x \) g for 20 min at 4°C.

To release the incorporated \(^{32}P\), the final precipitate was heated for 15 min at 100°C in 5.0 ml of a basic solution: 0.1 M NaOH, 0.2 M Na2CO3, 0.1 mM K2HPO4. When the solution cooled, 3.0 ml were assayed for \(^{32}P\) as described for measurement of hydrolysis of ATP.

**Measurement of ATP\(^{32}P\) Hydrolysis**

The method for measuring the rate of release of \(^{32}P\), ATP has been described previously (3). The reaction conditions were the same as those used for measuring phosphorylation except that the protein concentration was lower and the total volume was 10 ml.

**Conditions for Separately Measuring Activities of Site I and Site II**

*Site I, Na\(^{+}-K\(^{+}\) ATPase*—In general to determine this activity, the rate of ATP hydrolysis with and without Na\(^{+}\) was measured in the presence of high levels of ATP (e.g. 10\(^{-4}\) M) or high levels of K\(^{+}\) (e.g. 15 to 25 mM) or both. The activity at Site I is the difference between these rates with and without Na\(^{+}\). The specific conditions for particular experiments are given in the legends of the figures. At high levels of ATP, hydrolysis by Site II was negligible; in experiments in which the ATP concentration was lower, the level of K\(^{+}\) was high enough to inhibit Site II and again make its contribution negligible.

*Site II, Na\(^{+}\) ATPase*—In general to determine this activity, the rate of ATP hydrolysis with and without Na\(^{+}\) was measured in the absence of K\(^{+}\). The activity at Site II is the difference between these rates with and without Na\(^{+}\). The specific conditions for particular experiments are given in the legends of the figures. In the absence of K\(^{+}\), Site I does not hydrolyze ATP.

* Mg\(^{++}\) ATPase*—This is the ouabain-insensitive activity measured in the absence of monovalent cations.

**Determination of \(V_{\text{max}}\) and \(K_m\) at different temperatures**

\(K_m\) and \(V_{\text{max}}\) were determined from reciprocal plots of velocity against ATP concentration. At each temperature, the rates of hydrolysis were measured under optimal ion conditions for that temperature. Preliminary estimates made at each temperature gave the approximate \(K_m\) value. For the experimental determinations, substrate concentrations were then chosen to cover a range from 0.25 to 4 times the \(K_m\). For each determination of \(V_{\text{max}}\) and \(K_m\), six substrate concentrations were used and the reciprocal plots were linear.

**RESULTS AND DISCUSSION**

**Hydrolysis and Phosphorylation as Function of Na\(^{+}\)**—At any Na\(^{+}\) concentration, with K\(^{+}\) and ATP saturating, the rate of hydrolysis at Site I, \(v\), was directly proportional to the measured concentration of phosphorylated intermediate, \(E \sim P\).

\[
v = k_{\text{cat}} \cdot (E \sim P)
\]

where \(k_{\text{cat}}\) is the proportionality constant when the K\(^{+}\) level is optimal. From Equation 1 it follows that for any concentration of Na\(^{+}\)

\[
\frac{v}{V_{\text{max}}} = \frac{E \sim P}{E \sim P_{\text{sat}}}
\]

where \(E \sim P_{\text{sat}}\) is the concentration of complex when both Na\(^{+}\) and K\(^{+}\) are at optimal levels. This relationship is shown in Fig. 1. The experimentally calculated value for \(k_{\text{cat}}\) was 1.2 \( \times 10^4 \) min\(^{-1}\); \(E \sim P_{\text{sat}}\) was 125 pmoles per mg.

**Hydrolysis and Phosphorylation as Function of K\(^{+}\)**—At any concentration of K\(^{+}\), with Na\(^{+}\) and ATP saturating, the rate of hydrolysis at Site I was directly proportional to the concentration of unphosphorylated sites, \(E'\cdot\text{ATP}\), i.e. the difference between the total concentration of enzyme sites, \(E\), and the measured concentration of \(E \sim P\).

\[
v = k_{\text{cat}} \cdot (E'\cdot\text{ATP})
\]

where \(k_{\text{cat}}\) is the proportionality constant when the Na\(^{+}\) level is optimal, and

\[
E = E'\cdot\text{ATP} + E \sim P
\]

Furthermore, for any concentration of K\(^{+}\)

\[
\frac{v}{V_{\text{max}}} = \frac{[E'\cdot\text{ATP}]}{[E'\cdot\text{ATP}]_{\text{sat}}} = \frac{E'\cdot\text{ATP}}{E'\cdot\text{ATP}_{\text{sat}}}
\]

where \(E'\cdot\text{ATP}_{\text{sat}}\) is the concentration of unphosphorylated sites when both Na\(^{+}\) and K\(^{+}\) are at optimal levels. The relationship is shown in Fig. 2. The experimentally calculated value for \(k_{\text{cat}}\) was 0.65 \( \times 10^4 \) min\(^{-1}\); \(E'\cdot\text{ATP}_{\text{sat}}\) was 325 pmoles per mg.

As described below, \(E\) is obtained by measuring \(E \sim P\) in the absence of K\(^{+}\).
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FIG. 2. The effect of K+ concentration on hydrolysis of ATP by Site I and on the phosphorylation reaction. Conditions were the same as for Fig. 1 except the Na+ concentration was 100 mM and the K+ concentration was as indicated. $v/V_{\text{max}}$ is the rate of ATP hydrolysis at Site I relative to the maximum under saturating conditions; $E_{\text{ATP}}/E_{\text{ATP,sat}}$ (C) is the calculated concentration of unphosphorylated sites relative to the maximum under saturating conditions. The calculation of $E_{\text{ATP}}$ is described in the text. The K+ for K+ was 1 mM; $V_{\text{max}}$ was 1.6 pmoles per mg per min; $E_{\text{ATP,sat}}$ was 325 pmoles per mg.

FIG. 3. The effect of ATP concentration on hydrolysis of ATP by Site I and on the phosphorylation reaction. Conditions were the same as for Fig. 1 except for the indicated ATP concentration. The symbols are the same as for Fig. 1. The $K_0$ for ATP was $10^{-4}$ M.

rate of hydrolysis at Site I was directly proportional to the measured concentration of $E \sim P$

$$v = k_{\text{Na}} (E \sim P)$$

(6)

Here $k_{\text{Na}}$ was the same as in Equation 2, and, at any concentration of ATP

$$\frac{v}{V_{\text{max}}} = \frac{E \sim P}{E \sim P_{\text{sat}}}$$

(7)

as shown in Fig. 3.

Formation and Breakdown of $E \sim P$ as Steps in ATP Hydrolysis—These proportionality constants relating the hydrolytic activity of Site I to the phosphorylation reaction fit the generally accepted reaction scheme for the Na+-K+ ATPase

$$E + ATP \rightarrow E \cdot ATP \rightarrow E \sim P \rightarrow E + P_i$$

(8)

In this simple formulation, $k_{\text{Na}}$ and $k_{\text{K}}$ are taken as over-all rate constants; the designation $E_{\text{ATP}}$ includes all enzyme complexes other than the directly measured acid-stable enzyme-phosphate complex, and the designation $E \sim P$ includes all forms of the acid-stable complex.

With such a sequence of reactions, the following relationships hold in the steady state

$$v = k_{\text{Na}} (E_{\text{ATP}}) - k_{\text{K}} (E \sim P)$$

(9)

and these are the same relationships observed experimentally. Also from this equation and Equation 4, it follows that:

$$\frac{E \sim P}{E_{\text{sat}}} = \frac{k_{\text{Na}}}{k_{\text{Na}} + k_{\text{K}}}$$

(10)

and at saturating concentrations of ATP, Na+ and K+:

$$\frac{E \sim P_{\text{sat}}}{E} = \frac{k_{\text{Na}}}{k_{\text{Na}} + k_{\text{K}}}$$

(11)

With the experimentally determined values for these constants, the calculated ratio of $E \sim P_{\text{sat}}/E$ was 0.35.

This ratio was also determined more directly. $E \sim P_{\text{sat}}$ 125 pmoles per mg, was measured with Na+, K+, and ATP all at optimal levels. $E$, 450 pmoles per mg, was obtained by measuring $E \sim P$ with Na+ and ATP optimal but in the absence of K+. Under these conditions $E$ equals $E \sim P$ since $k_{\text{K}}$ in Equation 10 is now zero, and the ratio $E \sim P/E$ equals 1.0. The direct estimate of the ratio $E \sim P_{\text{sat}}/E$ in the steady state agreed with the calculated value, both being approximately one-third.

Thus, with optimal Na+ but no K+ to catalyze the breakdown of $E \sim P$, all of the sites are phosphorylated—450 pmoles per mg (this gives an apparent equivant weight of about 2,000,000 for the Na+-K+ ATPase). With Na+ and K+ at optimal concentrations and the formation and breakdown of $E \sim P$ in the steady state, only one-third of the total number of sites is in the form of $E \sim P$ at any given time.

Ion Dependence of $k_{\text{Na}}$ and $k_{\text{K}}$—In general, $k_{\text{Na}}$ is a function of the Na+ concentration and $k_{\text{K}}$ is a function of the K+ concentration. From measurements of $E \sim P$ and $E_{\text{ATP}}$, these functions were determined empirically from the steady state relationships. To determine how $k_{\text{K}}$ varied with the K+ concentration, $k_{\text{Na}}$ was set equal to $k_{\text{Na}}$, and $v$ and $E \sim P$ were measured at different K+ concentrations. At each K+ concentration, $k_{\text{K}}$ was calculated from:

$$v = k_{\text{K}} (E \sim P)$$

(12)

Fig. 4 shows how $k_{\text{K}}$ changed with the K+ concentration.

Similarly, to determine how $k_{\text{Na}}$ varied with the Na+ concentration, we set $k_{\text{K}}$ equal to $k_{\text{K}}$, and measured $v$ and $E \sim P$ at different concentrations of Na+. At each Na+ concentration, $k_{\text{Na}}$ was calculated from:

$$v = k_{\text{Na}} (E \cdot ATP) = k_{\text{Na}} (E \sim P)$$

(13)

Fig. 5 shows how this constant changed with the Na+ concentra-
Temperature Dependence of Hydrolysis by Different Sites—In past work (14, 15), there has been some difficulty in correlating hydrolysis and phosphorylation at low temperatures. Moreover, the effects of K+ at low temperature have been difficult to interpret (16, 17). It seemed to us that these problems might come from the failure to recognize the relatively high contribution that Site II, the Na+ ATPase, makes to over-all hydrolysis at low temperature. This led us to study each of the ATPases in calf brain microsomes separately as a function of temperature. The conditions for measuring each activity separately and the methods for determining \( V_{\text{max}} \) and \( K_m \) are described under “Materials and Methods.”

Fig. 6 shows how the \( V_{\text{max}} \) for Site I, Site II, and the Mg++ ATPase (background activity insensitive to Na+, K+, and ouabain) changed with temperature. A notable feature in these Arrhenius plots is the nonlinearity in the curve for Site I, the Na+-K+ ATPase. This curvature has been observed before in rabbit and rat brain microsomes even when total hydrolysis was measured without any attempt to distinguish Sites I and II (15, 18). The sharp falling off of the curve is not a reflection of cation or substrate inhibition of Site I at low temperature; at each temperature the measurements were made under optimal conditions for that temperature, and the reciprocal plots showed no evidence for inhibition at any temperature. Instead, there is probably a reversible, temperature-induced conformational change. This phenomenon has been reported for a number of other enzyme systems, e.g. Reference 9. For the Na+-K+ ATPase, between 25 and 35°, the energy of activation was 12 kcal per mole; between 1 and 11° it was 51 kcal per mole. For the Na+ ATPase over the entire temperature range the energy of activation was 19 kcal per mole; for the Mg++ ATPase it was 14 kcal per mole.

Fig. 7 shows the temperature dependence of \( K_m \) for each of the enzymes. The curve for Site I shows a break similar to that seen in the \( V_{\text{max}} \) curve (Fig. 6). Here again this probably reflects the temperature-induced conformational change affecting Site I.

Temperature Dependence of Phosphorylation Reaction—Comparing phosphorylation and hydrolysis at 9°, the same relationships were found to hold as those described for 37°; that is, the data at 9° fit the steady state relationships given in Equation 10. We conclude that hydrolysis at Site I occurs through the same mechanism at 9° as at 37°.

As shown in Fig. 8 there was no significant change in \( E \) or \( (E \sim P)_{\text{sat}} \) with temperature, and the ratio \( (E \sim P)_{\text{sat}}/E \) was constant. Since \( (E \sim P)_{\text{sat}}/E \) is a function of \( k_{\text{Na}^+} \) and \( k_{\text{K}^+} \) as given in Equation 12, the constancy of this ratio suggests that both these rate constants—for formation and breakdown

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**Fig. 4.** The effect of K+ concentration on \( k_{\text{K}^+} \). Conditions were the same as for Fig. 2. The calculation of \( k_{\text{K}^+} \) is described in the text.

**Fig. 5.** The effect of Na+ concentration on \( k_{\text{Na}^+} \). Conditions were the same as for Fig. 1. The calculation of \( k_{\text{Na}^+} \) is described in the text.

**Fig. 6.** Temperature dependence of \( V_{\text{max}} \) for the different ATPase sites. Conditions: 1 μg of protein per ml, 100 mM imidazole (pH 7.3) (adjusted for each temperature), 5 mM MgCl₂. Na+ and K+ concentrations were adjusted as described under “Materials and Methods” to allow the separate measurement of each activity. The ordinate gives the rate of hydrolysis in micromoles per mg per min. 〇, Site I; △, Site II; ●, Mg++ ATPase.
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The ordinate gives the concentration of ATPase sites. Conditions were the same as for Fig. 6. The determination of $K_m$ is described under "Materials and Methods." The ordinate gives $K_m$ in moles per liter. ○, Site I; △, Site II; ●, Mg$^{2+}$ ATPase.

**Fig. 7.** Temperature dependence of $K_m$ for the different ATPase sites. Conditions were the same as for Fig. 6. The determination of $K_m$ is described under "Materials and Methods." The ordinate gives $K_m$ in moles per liter. ○, Site I; △, Site II; ●, Mg$^{2+}$ ATPase.

**Fig. 8.** Temperature dependence of the phosphorylation reaction. Conditions were the same as for Fig. 2 except for the indicated temperature. ○, with 10 mM K$^+$/; this gives a measure of $E \sim P_{\text{sad}}$ as described in the text. ●, without K$^+$; gives a measure of $E$ as described in the text.

**Table I**

| [ATP] | [K$^+$] | Velocity$^a$ |
|-------|--------|-------------|
| 10 μM | 0 mM   | 1.05        |
| 10 μM | 15 mM  | 0.95        |
| 1 μM  | 0 mM   | 0.85        |
| 1 μM  | 15 mM  | 0.40        |
| 50 μM | 0 mM   | 1.05        |
| 50 μM | 15 mM  | 1.18        |

$^a$ Each value given is the average of two determinations; these duplicates agreed within 5%.

$E \sim P$—change proportionately. Moreover, they must both decrease when temperature is lowered because $V_{\text{max}}$, which equals $k_{\text{cat}}(E \sim P)_{\text{sat}}$, falls with temperature.

**Interpretation of K$^+$ Effects at Low Temperature**—Table I shows some typical effects of K$^+$ at low temperature. Observations of this kind have been reported for rabbit brain (15), pig brain (17), and human red blood cells (16). To interpret the apparently complex effects of K$^+$ on the ATPase at low temperature it is helpful to emphasize three points: (a) at lower temperatures under optimal conditions, Site II accounts for a greater proportion of the total ATP hydrolysis (Fig. 6); (b) Site II at all temperatures has a much lower $K_m$ (Fig. 7); and (c) K$^+$ inhibits Site II and activates Site I (3).

With these features in mind, consider for example the effect of K$^+$ at 0° and 10 μM ATP (Table I). With Na$^+$ but no K$^+$, all of the hydrolysis occurs at Site II. The addition of K$^+$ activates Site I and inhibits Site II. Since both sites have about equal activity under these conditions at 0°, these opposite effects are offsetting and the response is an apparent indifference to K$^+$.

At the lowest level of ATP in Table I (1 μM), Site I is far more unsaturated ($K_m = 2.5 \times 10^{-2}$ μM at 0°) than Site II ($K_m = 2.5 \times 10^{-4}$ μM at 0°). Under these conditions, the K$^+$ activation of Site I will not completely offset the inhibition of Site II. The over-all effect is therefore inhibition by K$^+$.

Thus, as shown by the examples in Table I, with these two enzymes operating independently, K$^+$ can activate, inhibit, or have no effect at all on the over-all rate.

**General Conclusions**—The evidence for two independent ouabain-sensitive ATPase sites in calf brain microsomes was presented by us in an earlier paper (3). Before this, Caerwicki et al. (2) had inferred the presence of a second ATPase from their kinetic studies on rat erythrocytes, and Kanazawa, Saito, and Tonomura (15) had obtained similar data from their work on rabbit brain microsomes, although they interpreted it somewhat differently. More recently, a number of reports have appeared, on electric organ (20) and kidney (21), which we believe support the argument for two Na$^+$-dependent enzymatic sites.

All of this, it seems to us that Site II, the Na$^+$-ATPase, as well as Site I, may have wide general distribution in biological membranes.

In any event, when Site II exists in a preparation, the recognition of its presence helps to analyze the kinetics of ATP hydrolysis. For example, in this paper, we have shown that the acid-stable enzyme-phosphate complex of calf brain microsomes is an intermediate in the mechanism of hydrolysis by Site I, not only at 37°, but at low temperature as well. Without recognizing Site II, the correlation of over-all hydrolysis with $E \sim P$ formation is not nearly so good at 37° and cannot be made at all when the temperature is low (11, 15). Also, as we have shown here and in the earlier paper (3), a consideration of two independent sites explains certain complexities in the kinetics of over-all hydrolysis and in the cation effects, particularly at low temperature.

The correlation between hydrolysis at Site I and the phosphorylation reaction adds further support to the proposal of Post et al. (13) that an acid-stable enzyme-phosphate complex of microsomal preparations is an intermediate in the hydrolysis of ATP. On the other hand, our data give no indication that
such an intermediate plays any role in the hydrolysis at Site II. The mechanism at this site, then, remains unknown.

In considering the biological function of Site II, there are many Na+-dependent membrane transport systems in which it could take part. One possibility that seems particularly promising is a Na+ pump in the red blood cell described by Hoffman and Kregnow (22). It has the following properties: (a) it is activated by external Na+, optimal levels being about 150 mM; (b) it transports Na+ from inside to outside the cell; (c) it is ATP-dependent; (d) it needs no K+; and (e) it is inhibited by ouabain, and this effect is antagonized by K+. For comparison, Site II is activated by the same high levels of Na+ that activate this pump, much higher levels than the Na+-K+ ATPase requires. It seems from this that Site II might be activated in vivo by external Na+. Also, similar to this Na+ pump, Site II needs no K+, is inhibited by ouabain, and—despite the fact that its activity requires no K+—the effect of ouabain is antagonized by K+. Whether or not Site II is actually a part of this Na+ pump described by Hoffman and Kregnow remains to be seen.

REFERENCES
1. Neufeld, A. H., and Levy, H. M., Fed. Proc., 29, 801 (1970).
2. Neufeld, A. H., and Levy, H. M., J. Biol. Chem., 244, 6493 (1969).
3. Czerwinski, A., Gitelman, H. J., and Welt, L. G., Amer. J. Physiol., 213, 786 (1967).

1 Unpublished experiments.
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