Elimination of the Hydroxyl Groups in the Ribose Ring of ATP Reduces Its Ability to Phosphorylate the Sarcoplasmic Reticulum Ca\textsuperscript{2+}-ATPase*

(Received for publication, August 25, 1992)

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2’-Deoxyadenosine 5’-triphosphate, 3’-deoxyadenosine 5’-triphosphate, and 3’-amino-3’-deoxyadenosine 5’-triphosphate were substituted for ATP in the Ca\textsuperscript{2+} pumping cycle of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase. The rate of phosphorylation of the enzyme decreased by more than an order of magnitude when either of the hydroxyl groups was eliminated from the ribose ring. This resulted in low rates of hydrolysis and low levels of phosphoenzyme intermediate. In addition, the $K_{cat}$ of hydrolysis and the $K_{m}$ of phosphorylation of the derivatives modified in the 3’ position were decreased by a factor of 5–10. Otherwise, the 3’-amino-3’-deoxyadenosine 5’-triphosphate was utilized in a manner equivalent to ATP. Because the observed rates of phoshoenzyme formation with the deoxynucleotides were lowered to the extent that they would be rate-limiting in the enzyme cycle, and the level of phoshoenzyme intermediate remained low when the enzyme was back-inhibited by high Ca\textsuperscript{2+} concentrations, it was concluded that the majority of the enzyme remained in a preliminary conformation, in which the phosphorylation reaction could not proceed although substrate and Ca\textsuperscript{2+} were bound. It was then proposed that, following Ca\textsuperscript{2+}-induced changes in conformation, the hydroxy groups are able to form hydrogen bonds with pertinent segments of the phosphorylation domain, helping to stabilize an enzyme-substrate complex, one function of which may be to provide the proper stereochemistry for phosphate transfer.

The SR\textsuperscript{2} Ca-ATPase transports Ca\textsuperscript{2+} against a gradient into the interior of the reticulum, and thus regulates Ca\textsuperscript{2+} concentration in the cytoplasm of the muscle cell. The binding of 2 Ca\textsuperscript{2+} ions to high affinity sites on the cytoplasmic side of the ATPase activates the enzyme by promoting changes in structure, which, in turn, allow for the proper utilization of ATP. A key step in this process is the transfer of the ATP γ phosphate to an aspartyl side chain on the enzyme. This transfer triggers the occlusion of the bound Ca\textsuperscript{2+}, one of the first steps in the transport process. A subsequent change in the affinity for Ca\textsuperscript{2+} follows, and the bound Ca\textsuperscript{2+} is released to the interior of the reticulum in response to this change. The phosphoenzyme is then hydrolyzed, and the enzyme recycles (for reviews see Inesi et al. (1992), Jencks (1989), and MacLennan (1990)).

One of the most important and least understood aspects of this mechanism is the means by which Ca\textsuperscript{2+} binding controls the activation of the enzyme. A structural model has been derived from the primary sequence (Brandl et al., 1986), and it can be said with reasonable certainty that at least one, if not both, of the Ca\textsuperscript{2+} sites is located within a channel that spans the membrane (Clarke et al., 1989), a minimum of 45 Å from the nucleotide binding site (Scott, 1985), and certainly too far for Ca\textsuperscript{2+} to be directly involved in the mechanism of phosphorylation. It is also well established that the side chain which accepts the ATP γ phosphate (Asp-351) is on a very different portion of the peptide backbone than the residues that constitute the nucleotide binding site (Brandl et al., 1986). Aside from the constraints imposed by the necessity of the phosphate transfer to Asp-351, little is known about the topography of the active site, and certainly very little is known about the manner in which the different segments of the site must interact with the substrate to allow for precise control over phosphorylation.

It is our intention, here, to investigate the possibility that the ribose moiety of the ATP participates in the formation of an intermediate complex, formed between the substrate and the enzyme in response to Ca\textsuperscript{2+} activation in order to facilitate phosphate transfer. Evidence for such an intermediate comes from kinetic measurements (Petithory and Jencks, 1986; Stahl and Jencks, 1987), from changes in the rotational mobility of a spin-labeled sulphydryl residue (Cys-675), which shows that a conformational change occurs at precisely this step in the mechanism (Coan and Inesi, 1977; Coan, 1983), and from studies indicating that the properties of the metal that is chelated by the phosphates of the ATP affects the formation of a complex that directly precedes phosphate transfer (Chen et al., 1991; Hanel and Jencks, 1990).

Anderson and Murphy (1983) have demonstrated that 3’-deoxy-ATP and 2’-deoxy-ATP exhibit, respectively, a very low and a moderately low rate of turnover, and, in fact, all 3’ derivatives of ATP which have been used with the SR ATPase have been shown to turn over very slowly (Watanabe and Inesi, 1982; Dupont et al., 1982; Carvalho-Alves et al., 1985; Oliveira et al., 1988). With the deoxy derivatives, there are no
bulky moieties added to the ring that could cause steric hindrance, or present side reactions of their own, and the fact that the simple replacement of a hydroxyl group with a hydrogen has such pronounced effect suggests that these groups may have a specific role in the mechanism. Accordingly, we have prepared 2P derivatives of the deoxynucleotides and have pursued a series of experiments designed to isolate the steps in the mechanism that are dependent on the integrity of the hydroxyls. We have used 3'-NH2-ATP to compare the introduction of another polar moiety, which would also have the capacity to form a hydrogen bond, into the 3' position of the ribose ring.

MATERIALS AND METHODS

SR vesicles were prepared from the white skeletal muscle of rabbit kind legs by using methods previously described (Eletr and Inesi, 1972). Vesicles were stored in a buffered sucrose medium (30% sucrose, 10 mM MOPS, pH 6.8) at 4°C and were used within 4-5 days of preparation. Purified ATPase was prepared by method 2 of Meissner et al. (1973); ATP, phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, NADH, ionophore A23187, 3'-dATP (3'-deoxyadenosine 5'-triphosphate; cordycepin-5'-triphosphate), 2'-dATP (2'-deoxyadenosine 5'-triphosphate), and 5'-NH2-ATP (3'-amino-3'-deoxyadenosine 5'-triphosphate) were purchased from Sigma. ATP was obtained from Instituto de Pesquisas Nucleares (São Paulo, Brazil).

Protein concentrations were measured according to the techniques of Lowry et al. (1951) using bovine serum albumin as a standard, or from the absorbance at 280 nm using ε = 1.05 cm-1 mg-1 of SR protein in 1 ml of 1% sodium dodecyl sulfate (Thorley-Lawson and Green, 1973).

With purified ATPase, catalytic activity was measured by determination of the amount of 32P released in the medium after stopping the hydrolytic reaction with a 1:1 volume of a suspension of activated charcoal in 0.1 N HCl (Carvalho-Alveres et al., 1985). Three time points were taken at each analog concentration used, to insure linearity of the reaction. The reaction times and protein concentration were adjusted in such a way that the amount of 32P released in the steady state corresponded to a minimum of 15% and a maximum of 60% of the total nucleotide present at each concentration, thus significantly reducing the interference of 32P released from contaminant ATP (which represented a maximum of 2% of total nucleotide as measured independently; see below).

With SR vesicles, catalytic activity was measured with a coupled enzyme assay system with pyruvate kinase (8 μg/ml), phosphoenolpyruvate (1 mM), lactate dehydrogenase (220 μg/ml), and NADH (100 μg/ml) (Anderson and Murphy, 1983). The assay medium contained 10 mM MgCl2, 2 mM EGTA, 2 mM CaCl2, 100 mM MOPS, pH 6.8, 80 mM KCl, 1 pg/ml ionophore A23187, 5 mM ATP, and 0.01 mg/ml SR. With this system it was found that, when the nucleotide analog concentrations were kept below 300 μM, the measured velocities matched the velocities obtained by direct determination of 32P released from the analog, which indicates that the interference caused by hydrolysis and continuous regeneration of contaminant ATP was not significant.

The level of phosphorylase intermediate was determined by quenching the reaction with perchloric acid followed by washing of the acid-denatured protein on a Millipore filter, which was subsequently counted in a liquid scintillation counter (Ferreira and Verjovski-Almeida, 1988).

Preparation of γ-32P-Labeled Nucleotide Anals-—Anals were labeled with 32P at the γ phosphate by the method of Glynn and Chappell (1964) and were purified through a Dowex-1 column (Glynn and Chappell, 1964). The 3'-NH2-ATP analog did not bind efficiently to this resin, and it was purified on a DEAE-Sephadex column (Norby and Jensen, 1971).

To determine the amount of contaminant ATP in the γ-32P-labeled analogs, a sample of the labeled nucleotide was spotted on plastic-backed thin layer plates of cellulose with fluorescent indicator and developed with isobutyric acid, 0.5 N NH3 (5:5) (Guillory and Jeng, 1971). The plates were exposed to x-ray film, and the separated spots of ATP and ATP analog were cut out from the plates and counted in a scintillation counter. The contaminant ATP was found to be approximately 2% by this method. In addition, we requested that an analysis be performed by Sigma. Using high performance liquid chromatography to separate derivatives, they reported 1.5% ATP in their preparation of 3'-dATP.

RESULTS

The Nucleotide Concentration Dependence of Catalytic Activity—The catalytic activity of the SR ATPase with substrates that have been modified in the 2' or the 3' position of the ribose moiety is compared to that of ATP in Fig. 1a. The activity is given by the velocity of P1 production (using purified ATPase, closed symbols) and by the velocity of nucleoside diphosphate production (using intact SR vesicles, open symbols). The velocity of hydrolysis of 3'-dATP and of 2'-dATP is much lower than that of either 3'-NH2-ATP or ATP, with the effect being somewhat greater for the modification in the 3' position (data are reproduced on an enlarged scale in Fig. 1b). For each of the nucleotides, the data were fit with the sum of two hyperbolic functions, and best fit constants for the two K values and corresponding V max are given in Table 1. It is also evident that modification to the 3' position, but not the 2' position, lowers the K values. A lower K (at) is also observed with 3'-NH2-ATP, although the predicted values for V (at) and Vmax remain close to those of ATP.

With ATP as a substrate, the increase in state catalytic activity at higher substrate concentrations occurs without an increase in the level of steady state intermediates and is usually referred to as secondary activation. It is apparent in Fig. 1 that the 2'-dATP activity is similarly biphasic. However, a biphasic pattern is not clearly discernible with the 3' derivatives because of their lower affinity for the catalytic site, and the fit with two sets of constants was only slightly better than a fit with one set. Here, we chose to describe the data with two K and two V on the basis of the full set of experiments described below, as well as the data in Fig. 1. It should also be noted that the full concentration dependence of enzyme activity could only be obtained by direct measurement of P1 production. The more common method of measuring activity, which uses a regenerating system to monitor ADP production, could not be used with deoxynucleotide at concentrations above 500 μM because the regenerating system maintains traces of contaminant ATP (20% of Materials and Methods). This ATP will begin to compete for the catalytic site as the total concentration of nucleotide increases (see Fig. 7), which, in turn, leads to an overestima-
with SR vesicles (used by Anderson and Murphy and in the present study) to data obtained with purified ATPase. The titrations in Fig. 1 also compare data obtained with 3'-NH₂-ATP, and only the direct method could be used. This is not always the case with other substrates, such as ATP (Souza and de Meis, 1976) or UTP (Ferreira and Verjovski-Almeida, 1988), which show low levels of E-P during turnover but produce maximum levels when Ca²⁺ release is required to attain half of the maximal level of E-P, are close to the best fit values of $K_{V,1}$ for the velocity data.

Steady state levels of E-P are controlled by the net rate of phosphoenzyme formation as well as the subsequent rate of utilization of phosphoenzyme. With ATP as a substrate, maximum levels of E-P (close to saturation of the enzyme) are maintained because the slowest steps in the enzyme cycle involve the utilization of the phosphoenzyme for Ca²⁺ transport. This is not always the case with other substrates, such as ATP or UTP (Ferreira and Verjovski-Almeida, 1988), which show low levels of E-P during turnover but produce maximum levels when Ca²⁺ release is required to attain half of the maximal level of E-P.

### Table I

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$K_{V,1}$ (µM)</th>
<th>$V_{V,1}$ (µmol/min/mg)</th>
<th>$K_{V,11}$ (µM)</th>
<th>$V_{V,11}$ (µmol/min/mg)</th>
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<tbody>
<tr>
<td>3'-dATP</td>
<td>36 ± 10</td>
<td>0.19 ± 0.02</td>
<td>765 ± 200</td>
<td>0.55 ± 0.02</td>
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<tr>
<td>2'-dATP</td>
<td>4.3 ± 1</td>
<td>0.38 ± 0.04</td>
<td>250 ± 100</td>
<td>0.93 ± 0.04</td>
</tr>
<tr>
<td>3'-NH₂-ATP</td>
<td>25 ± 10</td>
<td>1.0 ± 0.2</td>
<td>3100 ± 500</td>
<td>2.0 ± 0.4</td>
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<tr>
<td>ATP</td>
<td>3.6 ± 1</td>
<td>1.0 ± 0.1</td>
<td>550 ± 100</td>
<td>2.4 ± 0.1</td>
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### Table II

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>$K_0$ (µM)</th>
<th>$E_{P, max}$ (µmol/mg)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (µM)</th>
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<tbody>
<tr>
<td>3'-dATP</td>
<td>31 ± 3</td>
<td>1.4 ± 0.1</td>
<td>5 ± 1</td>
<td>35 ± 0.2</td>
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<tr>
<td>3'-dATP + 10 mM Ca²⁺</td>
<td>1.4 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>35 ± 0.2</td>
<td>35 ± 0.2</td>
</tr>
<tr>
<td>2'-dATP</td>
<td>1.7 ± 0.3</td>
<td>0.92 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.2</td>
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<tr>
<td>2'-dATP + 10 mM Ca²⁺</td>
<td>1.0 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>1.9 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>3'-NH₂-ATP</td>
<td>57 ± 7</td>
<td>4.3 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>ATP</td>
<td>3.1 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>4.2 ± 0.2</td>
<td>4.2 ± 0.2</td>
</tr>
</tbody>
</table>

*From Verjovski-Almeida et al. (1978).*
is inhibited and the phosphoenzyme will not undergo hydrolysis. We have repeated measurements with 10 mM Ca\(^{2+}\) in the medium, a level which is known to inhibit release. However, we did not find that the level of E-P increased significantly (Table II). This not only indicates that the overall rate of phosphoenzyme formation is low, but that the molecular equilibria that favor high levels of E-P when turnover is inhibited are shifted to a non-phosphorylated species. The latter effect is not observed in ATP analogs where the adenosine moiety has been substituted but the ribose remains intact.

As an additional control, a time course was measured at 200 \(\mu\)M 3'-dATP to determine the duration over which maximal levels of E-P could be maintained during steady state turnover. Measurements of P\(_i\) production were made concomitantly. We found that maximal levels of E-P remained constant and could be maintained for at least 90 s, during which time P\(_i\) production remained constant with 60% of the substrate being consumed. This control is of particular importance because of the contaminant ATP in our preparations. Under similar circumstances, with TNP-ATP (a very tight binding substrate), measurable amounts of E-P were shown to be produced by ATP contamination, rather than the analog (Dupont et al., 1985; Bishop et al., 1987). In this case, however, P\(_i\) production was not constant and the early part of the time course reflected the concentration of ATP. The ability here to utilize 60% of the substrate while maintaining constant E-P shows that the phosphoenzyme is produced by utilization of 3'-dATP.

Rates of E-P Formation—At low substrate concentrations the rate of E-P formation can be measured and time courses are given in Fig. 3 for steady state conditions, and in Fig. 4 with high Ca\(^{2+}\) present. It is clear by inspection that the rate of formation is very low with both 3'-dATP and 2'-dATP, certainly much lower than that of ATP, which cannot be measured without rapid kinetic techniques. The rate of E-P formation with 3'-NH\(_2\)-ATP was also too rapid to be observed. Because of the lack of turnover, the data in Fig. 4 can be accurately described by the Michaelis-Menten equation, and the best fit values of the net rate constants and the dissociation constant that describe the full set of time courses at varying deoxynucleotide concentrations are given in Table II (the solid lines in the figure give the fit obtained with these constants). The measurements given in Fig. 3 could be taken concomitantly with the data in Figs. 1 and 2, but could not be accurately described by a net rate constant without taking into consideration the fraction of turnover at each concentration. To estimate a rate constant, comparable to the \(k_{net}\) obtained in Fig. 4, the velocity at each concentration given in Fig. 3 was corrected for the velocity of P\(_i\) production, and then by using the \(K_d\) estimated in our other experiments, a \(V_m\) and corresponding \(k_{net}\) could be calculated. The estimated values (0.5 s\(^{-1}\) for 2'-dATP and 0.6 s\(^{-1}\) for 3'-dATP) were close to those obtained in Fig. 4, and substantiate that the constants derived for the Michaelis-Menten fit hold for the steady state conditions. It should also be noted that in Fig. 4 the data points at 2 s consistently fall above the predicted curve. This can be attributed to the trace of ATP in the deoxy preparations, which is not rapidly hydrolyzed when high Ca\(^{2+}\) is present. The effect was even more apparent at times less than 1 s, at higher nucleotide concentrations, and limited the time over which data could be obtained. The important points to be noted here, however, are: 1) that the estimated rates of formation of the deoxy derivatives are approximately 2 orders of magnitude lower than that of ATP (\(k_{net} = 85\) s\(^{-1}\); Verjovski-Almeida et al. (1978)) and 2) the high degree of consensus observed with the different experimental approaches.

**Competition between 3'-NO'-ATP and 3'-dATP for the Catalytic Site of the ATPase**—As discussed above, the \(K_{m}\) of 3'-dATP is difficult to observe directly. Therefore, we measured competitive inhibition between the binding of a spinlabeled ATP and 3'-dATP (Fig. 5). The spectral parameters of the bound spin-label (3'-NO'-ATP; a pyrroline group in the 3' position) are indicative of highly constrained molecular motion, and the low field lines in the EPR spectrum can be clearly separated from the isotropic signal generated by the unbound fraction of the label (Oliveira et al., 1988) (see Fig. 5, inset). The bound signal was first followed as a function of 3'-dATP concentration to obtain a \(K_d\) for the spin-labeled nucleotide under the given conditions and establish the binding stoichiometry (Fig. 5a). Increasing amounts of 3'-dATP were then added and the decrease in bound signal was followed as a function of 3'-dATP concentration to obtain an apparent binding constant for the deoxy derivative (Fig. 5b). Similar results could be obtained by measuring the increase in signal of the free label. The apparent \(K_d\) of the 3'-dATP we obtained was 27 \(\mu\)M.

The complete displacement of the 3'-NO'-ATP (binding stoichiometry 4.4 nmol/mg) clearly demonstrates that the 3'-dATP saturates the catalytic site. It should also be noted that very high enzyme concentrations are used in EPR measurements (approximately 250 \(\mu\)M, as determined by the catalytic
The concentration of phosphoenzyme was measured with 10 mM CaCl₂ in the medium at the following concentrations. Panel a, [³²P] 3'-dATP: ○, 2 μM; △, 4 μM; □, 10 μM; ▽, 20 μM. Panel b, [³²P] 3'-dATP: ○, 0.5 μM; △, 1 μM; □, 2 μM; ▽, 4 μM; ◊, 8 μM. Solid lines give the fit to the data using the Michaelis-Menten equation and the constants given in Table II. Measurements were made at 22 °C.

Secondary Activation by 3'-dATP and 3'-NH₂-ATP—On the basis of the experiments presented above, we have predicted a biphasic concentration dependence for catalytic activity, which would mean that these derivatives are able to induce the secondary phase of activation. We designed an experiment to determine if these derivatives can activate ATP hydrolysis (Fig. 6). 100 μM ATP was first added, as this concentration should saturate the catalytic site but not stimulate the secondary phase. Increasing amounts of 3'-dATP or 3'-NH₂-ATP (100 μM to 1 mM) were then added and the effect on enzyme activity monitored, using the regenerating system to maintain constant concentrations of the nucleotides. As is evident in the figure, the curve was nearly the same as when completed with ATP (solid line). At deoxyntide concentrations above 1 mM inhibition of hydrolysis was observed as the analog competed with ATP for the catalytic site.

FIG. 5. Competition between 3'-dATP and 3'-NO-ATP for the catalytic site of the ATPase. a, binding of 3'-NO-ATP to the ATPase is given as a function of total 3'-dATP concentration. The solid line gives the best fit to the data with $K_d = 180 \mu M$ and a saturation level of 4.4 nmol of 3'-NO-ATP/mg of SR protein. The inset shows the low field lines in the EPR spectrum. The bound 3'-NO-ATP generates the broad, anisotropic component, and the free tumbling fraction of 3'-NO-ATP generates the sharp isotropic component. The decrease in the peak height of bound spin-label (A to B, respectively) and the concomitant increase in the free label peak height (C to D, respectively) are shown for the addition of 200 μM 3'-dATP to a solution containing 60 mg/ml SR and 900 μM 3'-NO-ATP. b, the decrease in the EPR spectrum of 3'-NO-ATP (7, 900 μM; ◊, 1800 μM total concentration) as a function of total 3'-dATP concentration. Solid lines give the fit to the data with a 3'-dATP $K_d$ of 27 μM and the 3'-NO-ATP parameters given above. To obtain parameters, concentrations of free 3'-NO-ATP (in a) and free 3'-dATP (in b) were estimated from initial estimates of the respective $K_d$ and of the maximum nucleotide bound, and best fit values were determined by iteration. Measurements were made at room temperature.
Elimination of the hydroxyl groups in the ribose ring of ATP renders a very poor substrate for the SR Ca2+-ATPase. The major effect appears to be on the ability of the enzyme to accept the γ phosphate from the bound substrate. This is evident in a low rate of phosphoenzymatic formation, a low level of steady state intermediate, and, subsequently, a decrease in the rate of turnover.

The only observed effect of the modification that is not related directly to phosphoenzymatic formation is a lower binding affinity for the derivatives that are modified in the 3' position. Anderson and Murphy (1983) also found the binding affinity of 3'-dATP to be reduced but predicted a higher Km(1) (270 μM) on the basis of a fit with a single set of constants. We found that velocity data for the 3' derivatives (Fig. 1) could be fit with one set of constants as well as with two sets because of the relatively small difference in Km(1) and Km(2).

However, by incorporating 32P into the derivatives we were able to show that only the constants produced by the biphasic fit could account for the concentration dependence of phosphoenzymatic formation. Moreover, the higher Km(1) could not account for binding competition with the spin-labeled ATP (Fig. 5) or for the inhibition of hydrolysis of the ATP that we added to the samples shown in Fig. 7.

It is clear that our analysis has focused on the steps leading to the formation of the phosphoenzymatic, rather than those associated with transport of the bound Ca2+, or the subsequent hydrolysis of the phosphoenzymatic. Once the phosphoenzymatic is formed, steps concerning its utilization are first order (i.e. Reactions 3 and 4 below) and are generally found to be independent of the substrate, as is exemplified by studies with acetyl phosphate (Teruel et al., 1987; Bodley and Jencks, 1987). To look at the effect of ribose modification on these steps, we can compare Vcat, the rate of turnover per unit of phosphoenzymatic. Values for Vcat are given in Table III, using both Vcat(1) and Vcat(2) for the calculation. These values are within a factor of 2 of the Vcat of ATP.

When V2 is used to calculate Vcat, secondary activation by the substrate is taken into account. In Table I it is apparent that Vcat(2) is approximately twice Vcat(1) for all derivatives, as it is for ATP. In Table III we see that this ratio is maintained, with the Vcat calculated from Vcat(2) being about twice those calculated from Vcat(1). We cannot be certain whether the site associated with secondary activation is a separate binding site (Coll and Murphy, 1985) or represents the replacement of ADP at the catalytic site after phosphorylation has occurred (Bishop et al., 1987; Oliveira et al., 1988; Champaill et al., 1988), but it would be the principle means by which a substrate would influence utilization of the phosphoenzymatic. Considering that the ratio remains constant, as well as the fact that the 3' derivatives can activate ATP hydrolysis (Fig. 6), it does not appear that ribose modification effects secondary activation.

The main advantage to studying deoxynucleotides is that the modification to the natural substrate is minimal and

### Table III

The rate of turnover per unit of phosphoenzymatic

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Vcat(1)</th>
<th>Vcat(2)</th>
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<tbody>
<tr>
<td>3'-dATP</td>
<td>0.14</td>
<td>0.39</td>
</tr>
<tr>
<td>2'-dATP</td>
<td>0.16</td>
<td>0.40</td>
</tr>
<tr>
<td>3'-NH2-ATP</td>
<td>0.23</td>
<td>0.46</td>
</tr>
<tr>
<td>ATP</td>
<td>0.24</td>
<td>0.57</td>
</tr>
</tbody>
</table>

NTP is very low may be an indication that the 3'-dATP is not utilized as rapidly by the regenerating system as the ATP. It should be noted, however, that a Km(1) much higher than that given in Table I (i.e. one predicted by a fit with a single set of constants) would predict the burst at a much lower concentration of added nucleotide.

When measurements of 32P production were made with the additional ATP, there was no observed effect on the steady state catalytic activity, indicating that the ATP was rapidly consumed.
highly specific. From the data presented here, it can be concluded that the hydroxyl groups play an important role in the phosphorylation of the enzyme. A question then arises as to the nature of that role. The phosphorylation reaction can be described by a minimum of two steps, a conformational change followed by acylphosphate bond formation.

\[ E \cdot Ca^2+ + MgATP \rightarrow MgATP \cdot E \cdot Ca^2+ \]

(Reaction 1-4)

In Reaction 2, acylphosphate bond formation is thought to be rapid, with the conformational change \((E \rightarrow \varepsilon E)\) being the rate-determining step (Petithory and Jencks, 1986; reviews by Jencks, 1987). Ca++ binding also induces a conformational change in the enzyme (Dupont, 1976; Murphy, 1976; Champeil et al., 1978), but this change is distinct from that described in Reaction 2, which follows the further addition of substrate. The apparent binding constants that we measured would be represented by \(K_{40}\), whereas the observed rate of phosphoenzyme formation, in the absence of turnover, would be a function of forward and reverse rate constants associated with \(k_i \) and \(k_a\). Aside from the noted effect on binding constants, ribose modification could potentially alter any or all of these rate constants. However, considering both the low rate of phosphoenzyme formation and the low level of phosphoenzyme intermediate, even when the enzyme is back-inhibited by high Ca++, it is highly probable that the ability of the enzyme to maintain the \(\varepsilon E\) conformation has been altered.

Placement of the ribose interaction at this step in the enzyme mechanism comes from the knowledge that we have to date on the utilization of ATP, as well as the nature of the chemical mechanisms involved. Although the slowest steps in the pumping cycle are those associated with the utilization of the phosphoenzyme for transport (Petithory and Jencks, 1986) leading to the buildup of E-P during steady state turnover, when just the steps leading to phosphoenzyme formation are considered, not only is \(k_{-i}\) thought to be slower than \(k_p\), but \(k_a\) has been shown to be the rate-limiting constant in ATP production from MgADP. \((E - P - Ca^{2+})(side))\), whether the phosphoenzyme was initially formed with ATP (Pickart and Jencks, 1982) or acetyl phosphate (Teruel et al., 1987). This slow rate of reversal leads to a \(K_r > 1\). On the other hand, it has been estimated that \(K_r\) is between 0.3 and 0.8 (see Fernandez-Belda and Inesi (1986) and Hanel and Jencks (1990), respectively). Thus, even under ideal conditions the formation of the acylphosphate bond, by itself, would not be expected to stabilize the phosphoenzyme, and it is the combined high binding affinity of ATP and the stability introduced by the conformational change which act in concert with the slow steps in the cycle to maintain high levels of E-P.

With the deoxy derivatives, when the enzyme is inhibited by high Ca++, 20-30% of the enzyme is in the form of a phosphoenzyme. We know that under the conditions where we observe maximum E-P the substrates are saturating the enzyme, and therefore it can be assumed that most of the enzyme resides in the states described above as MgATP-E-Ca⁺ and MgATP-E-Ca₂⁺. We also know that the net rates of formation for both deoxy derivatives are very low (Table II), low enough to be rate-limiting in the cycle, and this would certainly predict that the majority of the enzyme would be held in preceding states. If the rate constants associated with acylphosphate bond formation were limiting, then the predominant species would be MgATP-E-Ca⁺. However, with ATP, acylphosphate bond formation is a rapid reaction, and the reaction mechanism would have to be greatly altered to be rate-limiting in the enzyme cycle. Considering that the ribose is fairly distal from the \(\gamma\) phosphate and that there are no indications of interaction between the two moieties, this would be very unlikely. It is also unlikely that elimination of the hydroxyl groups on the bound substrate would slow macromolecular movements of the enzyme per se, but if interactions between the ribose and the enzyme were required to stabilize MgATP-E-Ca⁺, then their elimination would shift the equilibrium toward MgATP-E-Ca₂⁺. The net rate constant we measured is a function of both the forward and the reverse rate constants \((k_{p1} \text{ and } k_{p2})\), and in this case the result would likely be a fast reverse rate.

Moreover, it is well established that formation of an acylphosphate from a carboxylate group and ATP is not an energetically favorable reaction in aqueous media (note that for ATP \(K_r\) is less than one). Pickart and Jencks (1984) pointed out that noncovalent binding of substrate moieties could make the reaction feasible, but also pointed out that for the SR Ca-ATPase the observed difference in binding energy between ATP and ADP would not be sufficient. It was then suggested that noncovalent binding of the phosphate moiety, which would occur at a subsequent step, could provide the needed free energy. Likewise, interactions of the ribose ring at the steps described in Reaction 2 would not contribute to a free energy term calculated from the initial binding constant of ATP, but would certainly make a contribution to the net free energy of phosphoenzyme formation. A link between pertinent segments of the catalytic site would also decrease the entropy of the system, making the overall reaction more feasible. It is quite reasonable to assume that a linkage through a hydroxyl group would be in the form of a hydrogen bond, and for this reason we preformed all pertinent experiments with a NH₂ group substituted into the 3' position. With the exception of a higher \(K_{40}\), this derivative appeared to function as well as ATP.

From structural standpoint the possibility of linking two sections of the catalytic site through the ribose moiety is very intriguing. As was discussed above, ATP is initially directed to a substrate binding site, a putative mononucleotide fold on the B cryptic fragment (Taylor and Green, 1989). Very specific changes must then occur to exercise precise control over phosphate transfer. Our data indicate that ribose interactions stabilize a conformation required for phosphate transfer. At this point, it seems quite likely that, in response to more global changes induced by Ca++ binding, the ribose may be able to attach to a site in the environs of Asp-351, linking the major sections of the catalytic site, and may provide the proper stereochemistry for the phosphate transfer.

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Utilization of Deoxy-ATP Analogos by the SR Ca$^{2+}$-ATPase


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