The sarcoplasmic reticulum of skeletal muscle functions physiologically in the regulation of muscle contraction by controlling transient changes in the cytosolic concentration of calcium. In vitro preparations of SR consist of closed vesicles containing a membrane-bound ATPase that couples Ca\(^{2+}\)-activated hydrolysis of MgATP to the transport of Ca\(^{2+}\) into the vesicles. Kinetic studies of the SR ATPase reveal a complex (biphasic) dependence of enzyme rate on MgATP concentration (1–3). CaATPase activity increases over a wide range of substrate concentrations (0.1 \(\mu\)M to 10 mM) and shows two apparent affinities for ATP (4). Although this effect may be attributable to a single catalytic site existing in two interconvertible forms (as has been postulated for the (Na,K)-ATPase (5)), or to two catalytic sites functioning alternately (as suggested for the F\(_1\)-ATPase (6, 13)), these mechanisms appear to be ruled out for the SR ATPase in light of the activating effects of some ATP analogs (7, 8). The activation of the enzyme by adenyl-5'-yl methylene diphosphonate, for example, is most directly explicable by a mechanism involving distinct catalytic (high affinity) and effector (low affinity) sites (8).

While the physiological substrate of the enzyme is MgATP (4), other phosphorylated compounds, including other nucleoside triphosphates (e.g., GTP and ITP) and p-nitrophenyl phosphate, can also serve as substrates for enzymatic hydrolysis coupled to Ca\(^{2+}\) uptake by SR vesicles (9, 10). Given this variety of phosphorylated compounds hydrolyzed by the SR CaATPase, its substrate specificity appears to be quite low (4). However, all these pseudosubstrates are hydrolyzed more slowly than is ATP. As part of a general study directed toward delineating the structural features of calcium transport by the SR ATPase, this report describes significant changes in kinetic parameters which we find result from structural variations of the substrate at the 2'- and 3'-positions.

MATERIALS AND METHODS

The ATP analogs 2'dATP, 3'dATP, ddATP, araATP, 2'O-MeADP, and 3'O-MeATP were obtained from P-L Biochemicals. ATP, NADH, pyruvate kinase, phosphoenolpyruvate, and lactate dehydrogenase were purchased from Sigma Chemical Co. The calcium ionophore A23187 was obtained from Calbiochem. The other chemicals used were at least reagent grade.

Sarcoplasmic reticulum was isolated in vesicular form from rabbit white skeletal muscle by the method of Eletr and Inesi (11) and stored at 0°C in 10 mM Mops, pH 7.0, and 0.88 M sucrose for use within 4 days. Protein concentration was measured by the biuret method (12).

CaATPase activity was measured at 37°C by an assay that coupled ATP hydrolysis to NADH oxidation. The assay medium (volume = 1 ml) consisted of 50 mM Mops, pH 7.0, 75 mM KCl, 5 mM MgCl\(_2\), 1 mM EGTA, 1 mM CaCl\(_2\), 0.4 \(\mu\)g of A23187, 10 \(\mu\)g of SR protein, 0.2 mM phosphoenolpyruvate, 3.3 IU of lactate dehydrogenase, 17 IU of pyruvate kinase, and 0.1 mg of NADH. The assay solution lacking substrate was incubated at 37°C for 7 min, then ATP or analog was added to start the reaction, and the absorbance at 340 nm measured spectrophotometrically was recorded continuously. Enzyme activity was calculated from the slope of the linear portion of each trace, using a value of 6,200 M\(^{-1}\) cm\(^{-1}\) for the extinction coefficient of NADH. Since MgATP is the true substrate of the SR CaATPase (4), for each assay MgCl\(_2\) was always in 5 mM excess of the nucleotide concentration. The presence of A23187 in the assay medium rendered the SR vesicles freely permeable to Ca\(^{2+}\), so the measured ATPase activities represented steady state rates in the absence of a Ca\(^{2+}\) concentration gradient across the membranes.

For each substrate tested, doubling the SR protein concentration doubled the observed rate of NADH oxidation, demonstrating that only the concentration of CaATPase was rate-limiting. These control measurements were especially important for ATP analogs that were poor substrates of the CaATPase, since they demonstrated that the system of enzymes (in particular pyruvate kinase) coupling analog hydrolysis to NADHoxidation was not adversely affected by the analog in question. This procedure also indicated that the diphaso
forms of the analogs were efficiently rephosphorylated to their triphospho forms by pyruvate kinase. For measurements of 2′OMeATPase activity, the assay medium, lacking CaCl₂, contained P-enolpyruvate and NADH at double their usual concentrations. The diphospho form of the analog (2′OMeADP) was then added, and its conversion to 2′0MeATP by phosphorylation was observed as a decrease in A₃₄₅. CaCl₂ was then added to start the assay. Before addition of substrate to the assay medium, a slow rate of NADH oxidation was observed. This background activity was equivalent to a specific activity of 0.1-0.3 µmol/mg/min and probably was due to the presence of endogenous ATP (or ADP) in the SR preparation. This background rate was subtracted from the total rate measured after the addition of substrate.

The kinetic data for nucleotide hydrolysis were computer-fitted by a nonlinear regression method (which minimizes χ²) to the following steady state velocity equation (cf. Refs. 7 and 8):

\[ v = \frac{V_1(S/K_m) + V_2(S^2/K_mK_i)}{1 + S/K_m + S^2/K_mK_i} \]

where \( V_1 \) and \( V_2 \) are the maximal velocities without and with regulatory site occupancy and \( K_m \) and \( K_i \) are the apparent Michaelis and regulatory site constants, respectively. The correlation coefficients obtained were greater than 0.98.

RESULTS AND DISCUSSION

Ribose-modified ATP analogs were examined for effectiveness as substrates of the CaATPase. Using analogs altered in the substituents at the 2′- and 3′-positions, we assessed the influence of the ribose hydroxyl groups on the steady state enzyme kinetics. Changes in the hydroxyl groups included deoxygenation, methylation, and epimerization. The results of these experiments are shown in the form of Eadie-Hofstee plots in Figs. 1 and 2. All of the analogs were less effective than ATP as substrates of the CaATPase. For the three cases where the structural variation was limited to the 2′-position, the biphasic kinetics characteristic of ATP was observed.

From the kinetic parameters listed in Table I, it can be seen that turnover (V₁) of araATP is comparable to ATP, but its apparent affinity for enzyme, particularly for the catalytic site (K₅₀ being more than 15-fold higher), appears to be weaker. Since the absence (2′dATP) or methylation (2′OMeATP) of the 2′-hydroxyl results in more modest (about 4-fold) increases in K₅₀, it would appear that the configuration at the 2′-position is unimportant for turnover and of considerable significance for catalytic site binding.

The results obtained using 2′dATP suggest that the 2′OH of ATP contributes significantly to the interaction of substrate and enzyme, presumably by hydrogen bonding. Since the presence of hydrogen-bond acceptor capability in the form of 2′OMeATP does not restore the parameters to those observed for ATP, function of the 2′OH as a hydrogen-bond donor would seem more likely.

The three analogs differing from ATP in the 3′-position exhibited little or no activity as substrates. For 3′OMeATP, no activity was detectable at concentrations up to 1 mM. The concentration dependence of the activity of 3′dATP and ddATP (Fig. 2, inset) displayed no activation at higher concentrations, and therefore the data were fitted with two parameters (Table I). The rates obtained with 3′dATP or ddATP were so low that the possibility of ATP contamination of the analog stock was considered. Given the relation between enzyme velocity and ATP concentration in Fig. 1, contamination of the order of 0.1% ATP in the ddATP or 3′dATP samples could have produced the rates observed. Therefore, the kinetic parameters reported in Table I for these substrates represent upper limits; the true values may be less. Since it is unlikely that any of the 3′ analogs have conformations significantly different from ATP, their inability to act as hydrogen-bond donors would be the most plausible basis for their inadequacy as substrates.

For many nucleotide-binding enzymes, hydrogen bonding has been implicated as a mode of interaction with the ribose moiety (14-17). In the absence of a ribose hydroxyl, weaker substrate binding would be expected, as appears to be reflected in the higher K₅₀ values reported here. Decreases in catalysis would seem to be less likely, since these groups are several atoms removed from the γ-phosphoryl moiety. In fact, how-

![Fig. 1. Dependence of SR CaATPase activity on the concentration of ATP and 2′dATP.](http://www.jbc.org/)

![Fig. 2. Dependence of SR CaATPase activity on the concentration of various ribose-modified ATP analogs.](http://www.jbc.org/)

**TABLE I**

<table>
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<th>Analogue</th>
<th>K₅₀ (µM)</th>
<th>V₁ (µmol/min/mg protein)</th>
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<th>Vᵢ (µmol/min/mg protein)</th>
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ever, a study of a number of nucleotide-utilizing enzymes (alcohol, lactate, and glyceraldehyde-3-phosphate dehydrogenases, all of which are known to interact with the adenosine ribose of NAD by hydrogen bonding) showed that, for the 2' and 3'-deoxy analogs, binding was relatively unaffected, while catalysis was significantly impaired (18). Since for these enzymes the adenosine ribose is even farther removed from the catalytic center than is its counterpart in ATP, our results, especially with the 3' analogs, are not without precedent. It is reasonable to suggest that, for the SR ATPase, the ribose hydroxyls are important for substrate alignment, i.e. for inducing optimal positioning of the γ-phosphoryl group with respect to the catalytic center.

Consistent with this suggestion are inferences drawn from NMR measurements of nucleotides bound to the catalytic and low affinity sites of the ATPase (19). For both sites, binding appears to produce similar conformation changes of the ribose moiety. In particular, the free nucleotide’s predominantly 2’-endo conformation of the S type is changed to a 3’-endo conformation of the N type and the gauche-gauche conformation about the C4’-C5’ bond is changed to gauche-trans or trans-gauche. Since enzyme-ligand perturbations would be mutual, these structural changes imposed by the enzyme are perhaps the counterpart of the influence of the ribose on catalysis which is revealed by the present study.

REFERENCES
Alterations in the structure of the ribose moiety of ATP reduce its effectiveness as a substrate for the sarcoplasmic reticulum ATPase.

K W Anderson and A J Murphy


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