Phosphorylation of Ca-ATPase of Sarcoplasmic Reticulum with Different Substrates*

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ATP and GTP as substrate for phosphorylation of sarcoplasmic reticulum ATPase are compared. Maximal levels of phosphoenzyme are between 4.5 and 4.8 nmol per mg of protein when either substrate is used provided that phosphoenzyme hydrolysis are strongly inhibited by high calcium concentration (20 mM) and low temperatures (0 °C). The maximal values obtained with GTP are lower than those previously reported.

It is shown that this difference is due to underestimation of the specific activity of labeled nucleotides used in previous studies, as revealed by UV absorption and HPLC analysis.

The dependence of the phosphoenzyme levels on calcium concentration, pH and temperature confirm previous findings indicating that ATP, but no GTP, accelerates the rate limiting step of the catalytic cycle.

Introduction

It is well known that the Ca^{2+} pump of sarcoplasmic reticulum vesicles utilizes free energy derived from ATP or other substrates such as ITP, GTP, acetilphosphate, *p*-nitrophenylphosphate. [1] The first step of the process of utilization of these substrates is phosphoryl transfer to an aspartil group of the enzyme, resulting in an acid stable acylphosphate [1–4]. The level of phosphoenzyme so formed has been used to estimate the enzyme sites present during the phosphorylation of Ca-ATPase [5]. However the levels reported in the bibliography are conflicting. Levels varying between 3 to 10 can be obtained, apparently depending on the type of substrate used [2–13, 15]. From our own laboratory we have reported values between 6 and 8 nmol/mg of protein using ITP [9–13] and GTP [2] as substrates, while in the same conditions, we obtained 4 to 4.5 nmol/mg of protein with ATP.

The present report shows that these differences are due to the quality and purity of the nucleotides used.

On the other hand, previously reported differences [2, 9, 10, 12–14] between temperature, Ca^{2+} concentration and pH dependences of enzyme phosphorylation with ATP and GTP are confirmed.

Materials and Methods

Leaky vesicles reconstituted from purified Ca^{2+} dependent ATPase were prepared from sarcoplasmic reticulum vesicles of rabbit skeletal muscle as described by MacLennan [15].

The nucleotides were obtained from Sigma Chemical Co.

^{32}Pi was obtained from the Brazilian Institute of Atomic Energy. The [γ-^{32}]ATP and [γ-^{32}P]GTP were prepared as described previously [16]. The concentration of the nucleotides solutions were determined by absorption, using the following molar extinction coefficients [17]: $E_{253} = 13\,700\ M^{-1}\,cm^{-1}$ for GTP and $E_{259}\ 15\,400\ M^{-1}\,cm^{-1}$ for ATP.

Samples of nucleotides were analyzed by ion pared reversal phase HPLC. Chromatography were performed using a Water liquid chromatograph equipped with a model 441 UV detector fixed at 254 nm and a radial compression module with a radialpack C-18 column (5 × 100 mm). The running conditions was: mobile phase: 5 mM tetrabutylammonium phosphate pH 6.9 + 23% V/V methanol,

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Abbreviations: HPLC, High Performance liquid chromatograph; P_i, inorganic phosphate.

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flux: 1.5 ml/min; temp.: 25 °C. The samples were dissolved in this mobile phase to a final concentration of 5 mM and 10–15 µl were injected.

Phosphorylation reactions were performed as previously described and corrected for non specific binding [18].

The protein concentration were measured by the method of Lowry *et al.* [19].

Results and Discussion

Eventhough the sodium salts of GTP and ITP undergo spontaneous hydrolysis at high rate (as compared to ATP), solutions of trinucleotides are in general prepared by weighing calculated amounts of nucleotides, without considering either spontaneous hydrolysis or change of hydration during shipping, handling and stocking of the samples. It is then possible that the actual concentrations of GTP and ITP are often less than expected due to significant breakdown of nucleotide triphosphates. In fact we determined by HPLC that GTP and ITP sodium salts stored in a deep freezer for four months contained as much as 20% of GDP or IDP and 4% of GMP or IMP (Fig. 1, c and d). This leads to underestimation of specific radioactivity, when these samples are traced with [γ-^{32}P]trinucleotides. As a consequence the phosphoenzyme levels obtained with such samples of GTP and ITP are overestimated.

To avoid this problem, a litium salt of GTP (which is more stable than sodium salt) was used in the experiments reported here. Furthermore, it was determined by HPLC that the GTP solutions used in this experiments contained a maximum of 4% of GDP, and no GMP (Fig. 1b). Hydrolysis of ATP was found to be less than 0.5% (Fig. 1a).

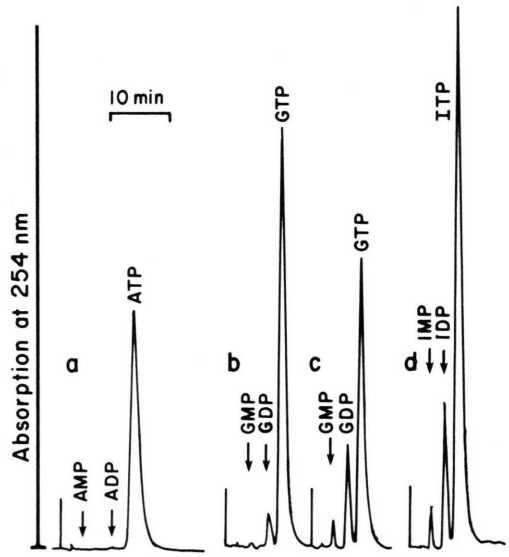

Fig. 1. HPLC of nucleotides samples. The samples were chromatographed as indicated in methods. a) ATP, sodium salt; b) GTP, litium salt; c) stocked GTP sodium salt; d) stocked ITP sodium salt. In all the cases 10 µl containing 40–60 nmols were injected. The elution times of the mono-, di- and triphosphonucleotides are indicated.

The maximal phosphoenzyme levels obtained with ATP and GTP

The steady state phosphoenzyme levels depend of both the velocity of phosphorylation and the velocity of hydrolysis. In order to obtain maximal levels of phosphoenzyme, hydrolysis was inhibited by decreasing the temperature and by increasing the Ca^{2+} concentration to the milimolar range [1–3, 10, 11]. When ATP was used as substrate the steady state levels varied between 4 to 4.5 nmol/mg of protein regardless of weather 0.1 or 20 mM Ca^{2+} were used at both 0 °C and 30 °C (Table I). When GTP was used, the phosphoenzyme levels varied between 4.5 to 4.8 nmol/mg of protein only if the Ca^{2+} concentration was sufficiently high to saturate the low affinity calcium binding sites ("back inhi-

Table I. Temperature and Calcium Concentration Dependence of the Phosphoenzyme Levels Obtained from ATP and GTP. Phosphoenzyme levels were obtained incubating purified ATPase with 0.5 mM [γ-^{32}P]ATP or [γ-^{32}P]GTP, in the presence of 50 mM Tris-maleate buffer, pH 7.4; 5 mM $MgCl_2$ and the indicated calcium concentrations. The protein concentration were 0.4–0.6 mg/ml. Incubation was carried out for times required to obtain maximal steady state levels of phosphoenzyme in each set of conditions: 5s (ATP) or 20s (GTP) at 30 °C, and 30s (ATP) or 120s (GTP) at 0 °C.

Temperature [°C]	Substrate	$[Ca^{2+}]$ [mM]	Phosphoenzyme[a] [nmol/gm of prot.]
30	ATP	0.1	3.79 ± 0.10
30	ATP	20	4.03 ± 0.06
0	ATP	0.1	4.39 ± 0.06
0	ATP	20	4.43 ± 0.07
30	GTP	0.1	2.44 ± 0.04
30	GTP	20	4.50 ± 0.12
0	GTP	0.1	3.39 ± 0.15
0	GTP	20	4.45 ± 0.10

[a] The values represent mean minus plus standard error of four independent experiments.

Table II. Effect of pH on the phosphoenzyme level. The purified ATPase was phosphorylated at 0 °C as indicated in Table I at pH 7.0 or 8.5. The values represent means plus minus standard errors. The number of independent experiments is indicated into the parenthesis.

Substrate	$[Ca^{2+}]$ [mM]	pH	Phosphoenzyme [nmol/mg of protein]
ATP	0.1	7.0	4.37 ± 0.08 (4)
ATP	0.1	8.5	4.57 ± 0.11 (4)
ATP	20	7.0	4.54 ± 0.12 (4)
ATP	20	8.5	4.54 ± 0.11 (4)
GTP	0.1	7.0	3.02 ± 0.09 (6)
GTP	0.1	8.5	4.80 ± 0.15 (6)
GTP	20	7.0	4.73 ± 0.17 (8)
GTP	20	8.5	4.72 ± 0.15 (8)

bition") (Table I and II). However, when calcium was present at concentrations saturing only the sites of high affinity (μM) the levels of phosphoenzyme formed by GTP were less than 4 nmol/mg of protein (Table I). This difference between GTP and ATP was already reported [2, 3, 9, 10–14] and attributed to an accelerating effect of ATP (but not of GTP) on a rate limiting step of the reaction cycle following phosphoenzyme cleavage and facilitating rephosphorylation and accumulation of maximal level of phosphoenzyme. This difference of the phosphoenzyme levels obtained with GTP and ATP have not been observed at high Ca^{2+} concentrations because the hydrolysis is too low to allow significative accumulation of dephosphorylated enzyme [2, 3, 9, 10, 12–14].

As the rate of phosphoenzyme hydrolysis has been shown to be pH dependent, we also studied the effect of pH on phosphoenzyme levels. The values obtained at pH 7.0–8.5 were similar to the previously obtained at pH 7.4 (Table II). At pH 8.5 the affinity of calcium binding sites is increased and 0.1 mM Ca^{2+} become sufficient to inhibit phosphoenzyme hydrolysis [11]. Accordingly high levels of enzyme phosphorylation can be observed.

In conclusion, we have falled to observe significative differences in the maximal levels of phosphoenzyme intermediate formed with ATP or GTP as substrates. Therefore, it is likely that previously reported differences in the maximal phosphoenzyme levels when various substrates are used, are due to inaccurate evaluation of specific radioactivity.

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