Phosphorylated intermediates of two hepatic microsomal ATPases

C. Ricky FLESCNER, Naomi KRAUS-FRIEDMANN and Gregory J. WIBERT

Department of Physiology and Cell Biology, University of Texas Medical School at Houston, Houston, TX 77025, U.S.A.

(Received 15 October 1984/Accepted 26 November 1984)

The hepatic microsomal Ca^{2+} - and Mg^{2+} -dependent ATPase phosphoenzyme intermediates were distinguished by using the chelators EGTA and CDTA (*trans*-cyclohexane-1,2-diamine-*NNN'*-tetra-acetic acid). The Ca^{2+} -ATPase intermediate is a hydroxylamine-labile base-labile 125 000- M_r phosphoprotein. The Mg^{2+} -ATPase intermediate is a hydroxylamine-stable base-stable 30 000- M_r phosphoprotein. This enzyme intermediate probably reflects the large basal ATPase activity of hepatic microsomal fraction. It is dependent on Mg^{2+} , since formation of the phosphoenzyme is abolished in the presence of CDTA. Under these conditions, the basal ATPase activity is dramatically decreased. These data demonstrate two separate and distinct enzymes which are responsible for the two ATPase activities of hepatic microsomal fraction. Furthermore, these data indicate that more meaningful data about the microsomal Ca^{2+} -ATPase might be obtained if the free ion concentrations are controlled with CDTA.

It is now well established that hepatic microsomal fraction (Moore *et al.*, 1975; Bygrave, 1978; Dawson, 1982), as well as that derived from other tissues (Robinson & Lust, 1968; Selinger *et al.*, 1970; Moore *et al.*, 1974; Bruns *et al.*, 1976; Colca *et al.*, 1983), are capable of ATP-dependent Ca^{2+} sequestration. This Ca^{2+} -sequestering activity has generated much interest because of its possible involvement in hormone action (Andia-Waltenbaugh & Friedmann, 1978; Bygrave, 1978; Andia-Waltenbaugh *et al.*, 1980). Associated with hepatic microsomal Ca^{2+} uptake is a Ca^{2+} -ATPase activity (Moore *et al.*, 1975; Dawson & Fulton, 1983; Famulski & Carafoli, 1982; Andia-Waltenbaugh *et al.*, 1980).

Studies on this Ca^{2+} -ATPase activity have been hampered by the presence of a large Ca^{2+} -independent ATPase activity present in the hepatic microsomal fraction (Andia-Waltenbaugh *et al.*, 1980; Dawson & Fulton, 1983). It is unclear if these two activities represent two enzymes, or if one enzyme is responsible for both activities. In order to address this problem, we have undertaken to examine the phosphorylated intermediate of the

Ca^{2+} -ATPase under various incubation conditions which alter the Ca^{2+} -ATPase and basal ATPase activities.

Data are presented below demonstrating that these two activities are associated with two different enzymes. Furthermore, by varying the incubation conditions, the activities of these two enzymes may be separated.

A preliminary account of this work has been presented (Fleschner *et al.*, 1984).

Experimental

Materials

ATP, EGTA, CDTA and Hepes were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ - ^{32}P]ATP (300–500 Ci/mmol) was obtained from ICN (Irvine, CA, U.S.A.). All other reagents were of the highest purity.

Microsomal preparation

The microsomal fraction was prepared from the livers of male fed Sprague–Dawley rats as described by Moore & Kraus-Friedmann (1983). Excised livers were minced and homogenized in ice-cold 250 mM-sucrose/10 mM-Hepes/0.2 mM- CaCl_2 , pH 7.2, with a motor-driven homogenizer fitted with a Teflon pestle. The homogenate (10%, w/v) was subsequently adjusted to 0.5 mM-EGTA.

Abbreviations used: Ca^{2+} -ATPase, Ca^{2+} -dependent ATPase; Mg^{2+} -ATPase, Mg^{2+} -dependent ATPase; CDTA, *trans*-cyclohexane-1,2-diamine-*NNN'*-tetra-acetic acid; SDS, sodium dodecyl sulphate.

The microsomal fraction was isolated by differential centrifugation as described by Moore *et al.* (1975). The homogenate was centrifuged at 1100g for 10 min in the SS34 rotor of a Sorvall RC2-B centrifuge. The resulting supernatant was centrifuged at 7700g for 20 min in the same rotor. The microsomal fraction was sedimented from the 7700g supernatant by centrifugation at 111000g for 60 min in the 42.1 rotor of a Beckman L5-50 ultracentrifuge. The microsomal fraction was resuspended in 120 mM-KCl/10 mM-Hepes, pH 6.8, to a final protein concentration of 20–30 mg/ml.

ATPase activity assay

ATPase activity was assayed in a medium containing 100 mM-KCl/20 mM-Hepes/1 mM-Na₃N, pH 6.8, and various other additions as described in the Figure legends. Total volume was 0.5 ml and protein was added to 1–1.5 mg/ml. The reaction was started by the addition of 2–4 mM-ATP. After incubation at 37°C for 15–30 min, the reaction was terminated by the addition of 2 ml of ice-cold 8% (w/v) trichloroacetic acid, and the P_i liberated was measured as described by Lanzetta *et al.* (1979).

Phosphorylation experiments

Phosphorylation of microsomal protein was performed in the same basic incubation medium described for ATPase activity, in a total volume of 0.5 ml. Other additions to the basic incubation medium were as described in the Figure legends. Microsomal protein (1–1.5 mg/ml) was incubated on ice for 5–10 s after the addition of 0.15–0.2 μM-ATP with [γ -³²P]ATP (100–500 Ci/mmol). The reaction was terminated by the addition of 2 ml of 20% trichloroacetic acid containing 0.2 mM-NaH₂PO₄. The trichloroacetic acid-precipitated protein was processed for SDS/polyacrylamide-gel electrophoresis.

Electrophoresis and autoradiography

Trichloroacetic acid-precipitated protein was solubilized in a sample buffer consisting of 25 mM-sodium phosphate (pH 5.5–6.0), 10% (v/v) glycerol, 2% (w/v) SDS, 10 mM-dithiothreitol and 0.00125% Bromophenol Blue (tracking dye). Samples were applied to a 3.5%-acrylamide stacking gel containing 25 mM-sodium phosphate, pH 5.5–6.0, and 0.1% SDS, and separated on a 5%-acrylamide resolving gel containing 75 mM-sodium phosphate, pH 5.5–6.0, and 0.1% SDS. Gels were electrophoresed at 35–40 mA/slab for 4–5 h at room temperature in a water-cooled electrophoresis chamber. The electrode buffer was 100 mM-sodium phosphate/0.1% SDS, pH 5.5–6.0.

After electrophoresis, gels were fixed for 10 min in 10% trichloroacetic acid in aq. 50% (v/v)

methanol and autoradiographed at –70°C for 2–4 days. After autoradiography, the gels were stained for protein with Coomassie Blue for 30 min, and destained overnight.

Calculation of free ion concentrations

Free metal ion concentrations were calculated by the method of Fabiato & Fabiato (1979) modified to run on a Hewlett-Packard 9845A computer (C. R. Fleschner, unpublished work). The absolute stability constants used were from Fabiato & Fabiato (1979) or Sillen & Martell (1971). The apparent stability constants at pH 6.8 used were (expressed as log *K_a*): CaEGTA, 6.003; MgEGTA, 1.377; CaCDTA, 6.226; MgCDTA, 5.696; CaATP, 3.603; MgATP, 3.957; K₂ATP, 0.557. The calculations included the endogenous Ca²⁺ and Mg²⁺ contents of the microsomal preparations as determined by atomic-absorption spectrophotometry as described by McDonald *et al.* (1977). The calculations also included the endogenous Ca²⁺ content of reagents as measured by Ca²⁺ electrode, which was calibrated as described by Bers (1982) with a standardized Ca²⁺ solution (Orion Research).

Other methods

Protein was assayed as described by Markwell *et al.* (1978), with bovine serum albumin (Sigma fraction V) as standard. Routine statistical methods were utilized, and included, where appropriate, Student's *t* test, paired or unpaired.

Results

Microsomal fractions isolated in our laboratory have been shown to be enriched in endoplasmic reticulum, as measured by glucose-6-phosphatase activity, and to contain little mitochondrial contamination, as measured by succinate dehydrogenase activity (Andia-Waltenbaugh & Friedmann, 1978). To prevent interference by this small amount (about 9%) of mitochondrial contamination, all experiments were performed in the presence of 1 mM-Na₃N, an inhibitor of the mitochondrial ATPase (Linnett & Beechey, 1979). The microsomal fraction also contains a small amount (about 10%) of plasma-membrane contamination, as measured by 5'-nucleotidase activity (Andia-Waltenbaugh & Friedmann, 1978). There is probably little interference by the plasma-membrane contamination in the present studies, since the ATP-dependent Ca²⁺ pump of rat liver plasma membrane has an alkaline pH optimum (Iwasa *et al.*, 1982; Kraus-Friedmann *et al.*, 1982), and has been found to be completely inactive at the pH used here (i.e. 6.8; Kraus-Friedmann *et al.*, 1982).

It is now generally accepted that hepatic endoplasmic reticulum contains a Ca^{2+} -ATPase, thought to be responsible for the ATP-dependent Ca^{2+} uptake by hepatic microsomal vesicles (Famulski & Carafoli, 1982; Dawson & Fulton, 1983; Andia-Waltenbaugh *et al.*, 1980; Moore & Kraus-Friedmann, 1983). Studies of the microsomal Ca^{2+} -ATPase have been hindered, however, by the large basal ATPase activity found in the absence of Ca^{2+} , which has previously been reported by us (Andia-Waltenbaugh *et al.*, 1980) and others (Dawson & Fulton, 1983). This phenomenon is clearly demonstrated by the data summarized in Table 1.

The data of Table 1 indicate that the basal microsomal ATPase is dependent on the Mg^{2+} concentration (or the MgATP concentration). It should be emphasized here that our reagents contain approx. $2\mu\text{M}$ - Ca^{2+} , as measured by Ca^{2+} electrode. In addition, the microsomal fraction as prepared in our laboratory contains approx. 20nmol of Ca^{2+} /mg of protein and 300nmol of Mg^{2+} /mg of protein, as determined by atomic-

absorption spectrophotometry. Under the conditions of Table 1 (i.e. in the presence of 2mM -EGTA or 2mM -CDTA), the free Ca^{2+} concentration is maintained at a very low value of $12\text{--}16\text{nM}$. For these experiments, the total ATP concentration was constant at 4mM (added as the potassium salt). Table 1 shows that increasing the Mg^{2+} concentration also increases the MgATP concentration. It is therefore impossible to determine if the increase in ATPase activity obtained by increasing the Mg^{2+} concentration is due to the increase in free Mg^{2+} concentration or to the increase in MgATP concentration.

The effect of a low concentration of free Ca^{2+} is demonstrated in Table 2. Under conditions where the free Mg^{2+} concentration and the MgATP concentration remained the same, an increase in the free Ca^{2+} concentration from 18nM to $0.9\mu\text{M}$ resulted in an increased ATPase activity. The increase in ATPase activity that is due to Ca^{2+} is significant ($P < 0.05$) as determined by Student's paired *t* test, and was $421 \pm 139\text{nmol}$ of P_i/h per mg of protein (mean $\pm$ S.E.M., $n = 3$).

Thus, as we (Andia-Waltenbaugh *et al.*, 1980; Moore & Kraus-Friedmann, 1983) and others (Moore *et al.*, 1975; Famulski & Carafoli, 1982; Dawson & Fulton, 1983) have previously demonstrated, low concentrations of free Ca^{2+} stimulate the ATPase activity of rat liver microsomal fraction. This Ca^{2+} -stimulated ATPase activity is tacitly ascribed to an ATPase separate from the basal Mg^{2+} -dependent ATPase. To our knowledge, no evidence has been published to substantiate this implication.

In order to determine if these two ATPase activities represent two distinct enzymes, experiments were undertaken to examine the phosphoenzyme intermediate(s). Phosphoproteins were formed by using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under incubation conditions favourable for phosphoenzyme intermediate formation. The phosphoproteins were then separated by SDS/polyacrylamide-gel electrophoresis and located by autoradiography.

The results of a typical experiment are shown in Fig. 1(a). In the absence of exogenous Ca^{2+} and in

Table 1. Mg^{2+} -dependence of microsomal ATPase activity

ATPase activity was determined as described in the Experimental section. The ionic composition of the incubation mixture was controlled with 2mM -EGTA (except where indicated by *, where 2mM -CDTA replaced EGTA), and various additions of MgCl_2 . The concentrations of ionic species were calculated as described in the Experimental section. Under these conditions, the free $[\text{Ca}^{2+}]$ was maintained at $12\text{--}16\text{nM}$. The values given represent the means $\pm$ S.E.M. for the numbers of experiments given in parentheses.

Free $[\text{Mg}^{2+}]$ (mM)	$[\text{MgATP}]$ (mM)	ATPase activity (nmol of P_i/h per mg of protein)
0.0006*	0.07*	70 ± 64 (3)
0.018	0.43	877 ± 122 (3)
0.023	0.52	1134 ± 109 (4)
0.088	1.5	1799 ± 243 (4)
1.80	3.7	1886 ± 300 (4)

Table 2. Ca^{2+} stimulation of microsomal ATPase activity

ATPase activity was assayed as described in the Experimental section. The ionic composition of the incubation mixture was controlled with 2mM -EGTA and the addition of CaCl_2 to yield the free Ca^{2+} concentration noted. Ionic concentrations were calculated as described in the Experimental section. The total ATP concentration was 2mM (added as K_2ATP). The ATPase activity values given are the means $\pm$ S.E.M. for three separate experiments.

Free $[\text{Mg}^{2+}]$ (μM)	$[\text{MgATP}]$ (mM)	Free $[\text{Ca}^{2+}]$ (μM)	ATPase activity (nmol of P_i/h per mg of protein)
47	0.48	0.018	619 ± 200
48	0.48	0.9	1040 ± 261

Fig. 1. Autoradiogram of ^{32}P -labelled hepatic microsomal proteins

Phosphorylation, SDS/polyacrylamide-gel electrophoresis and autoradiography were performed as described in the Experimental section. Additions to the basic incubation medium were as follows. (a): Lane 1, 2 mM-EGTA, 0.1 mM- MgCl_2 ; lane 2, 2 mM-EGTA, 0.1 mM- MgCl_2 , 2.05 mM- CaCl_2 ; lane 3, 2 mM-EGTA, 1.1 mM- MgCl_2 ; (b): lanes 1–3, 2 mM-EGTA, 0.1 mM- MgCl_2 , 2.05 mM- CaCl_2 . In (a) the resultant free Ca^{2+} , free Mg^{2+} and MgATP concentrations were (respectively): lane 1, 18 nM, 0.6 mM, 0.16 μM ; lane 2, 75 μM , 0.6 mM, 0.15 μM ; lane 3, 18 nM, 1.6 mM, 18 nM. (b) Phosphoprotein stability: phosphoprotein samples were examined for this as described in the text. Lane 1, control; lane 2, 0.8 M-hydroxylamine; lane 3, 0.5 M-NaOH.

the presence of 2 mM-EGTA, only one major phosphoprotein was seen. From this and other similar experiments the M_r of this phosphoprotein was found to be 30000 ± 1000 (S.E.M., $n = 9$). As shown in Fig. 1(a), lane 3, increasing the Mg^{2+} concentration to 1 mM resulted in an essentially identical phosphoprotein pattern. When Ca^{2+} was added, a second, larger, phosphoprotein was found. The M_r of the Ca^{2+} -dependent phosphorylation was 125000 ± 3500 (S.E.M., $n = 11$).

Further evidence suggesting that the 125000- M_r phosphoprotein represents the Ca^{2+} -ATPase phosphoenzyme intermediate is provided by experiments to determine the phosphoprotein stability. The results of a typical experiment are shown in Fig. 1(b). The trichloroacetic acid-precipitated phosphoproteins were resuspended in 0.8 M-hydroxylamine/0.15 M-sodium acetate, pH 6.0, or in 0.5 M-NaOH. Controls were resuspended in 0.8 M-

NaCl/0.15 M-sodium acetate, pH 6.0. Samples were incubated at room temperature for 10 min. The protein was then precipitated with trichloroacetic acid and processed for SDS/polyacrylamide-gel electrophoresis and autoradiography as described in the Experimental section.

As shown in Fig. 1(b), the Ca^{2+} -dependent phosphoprotein is labile in hydroxylamine, and in NaOH. This is typical of the acyl-phosphate intermediate of ATPases. Thus the 125000- M_r Ca^{2+} -dependent phosphoprotein is assumed to represent the phosphoenzyme intermediate of the Ca^{2+} -ATPase.

Interestingly, the 30000- M_r Ca^{2+} -independent phosphoprotein is stable in hydroxylamine and in NaOH. This is indicative of a phosphoester phosphoprotein. However, in experiments with a chase of unlabelled ATP it was found that the Ca^{2+} -independent ^{32}P incorporation was rapidly (10 s) and completely abolished by the subsequent addition of unlabelled ATP (C. R. Fleischner & N. Kraus-Friedmann, unpublished work). Therefore this phosphoprotein may represent an enzyme intermediate of the phosphoester type, such as that of a phosphatase (Lazdunski *et al.*, 1971).

The dependence of the two phosphoenzyme intermediates on Mg^{2+} was examined by experiments such as that of Fig. 2, by utilizing the two chelators EGTA and CDTA. In Fig. 2, lane 1, the phosphorylation was performed in the basic incubation medium without any additions. The incubation medium plus microsomal fraction contains sufficient Ca^{2+} and Mg^{2+} to support phosphorylation of both enzymes. Under these conditions, the free Ca^{2+} and free Mg^{2+} concentrations were 22 μM and 0.5 mM respectively. The inclusion of EGTA, which dramatically lowers the free Ca^{2+} concentration, but has no effect on the free Mg^{2+} or MgATP concentrations, essentially eliminates the Ca^{2+} -ATPase intermediate (Fig. 2, lane 2). Addition of CDTA to the reaction mixture, to decrease both free Ca^{2+} and free Mg^{2+} , resulted in elimination of both ATPase intermediates (Fig. 2, lane 3). Under these conditions, the MgATP concentration is also greatly decreased (to 1 nM).

Interestingly, addition of Ca^{2+} (in the presence of CDTA) to yield about 1 μM free Ca^{2+} , did not stimulate the Ca^{2+} -ATPase intermediate as expected (Fig. 2, lane 4). As shown in Table 2, a substantial Ca^{2+} -ATPase activity was found at about 1 μM - Ca^{2+} . Thus the absence of the Ca^{2+} -ATPase intermediate in Fig. 2, lane 4, may represent a requirement for free Mg^{2+} or MgATP in excess of the concentrations present under these conditions. On the other hand, these low concentrations of free Mg^{2+} (2.5 μM) and MgATP (3 nM) are capable of partially restoring the Mg^{2+} -ATPase intermediate.

Adding Mg^{2+} in the presence of CDTA raised the free Mg^{2+} concentration to $93\mu M$ and the $MgATP$ concentration to $77\mu M$. This produced greater restoration of the Mg^{2+} -ATPase intermediate (Fig. 2, lane 5). The free Ca^{2+} concentration was increased to $0.3\mu M$, but little or no Ca^{2+} -ATPase intermediate was observed.

Supplementation of the incubation mixture with Ca^{2+} and Mg^{2+} in the presence of CDTA restores both ATPase intermediates (Fig. 2, lane 6). The

free Ca^{2+} , free Mg^{2+} and $MgATP$ concentrations under these conditions were $59\mu M$, $0.2mM$ and $0.1\mu M$ respectively. The Ca^{2+} -ATPase intermediate detected in Fig. 2, lane 6, is substantially less intense than that in Fig. 2, lane 1, even though the free Ca^{2+} concentration is greater. This may be due to the lower free Mg^{2+} concentration under the conditions of Fig. 2, lane 6; or the free Ca^{2+} concentration under the conditions of Fig. 2, lane 1 may have been overestimated as a result of the assumption that all of the endogenous Ca^{2+} of the microsomal preparation (measured by atomic-absorption spectrophotometry) was free Ca^{2+} .

The ATPase activity of hepatic microsomal fraction was also examined under similar incubation conditions, and the results of these experiments are summarized in Table 3. The addition of CDTA decreases the ATPase activity by approx. 84% compared with the addition of EGTA. Under these conditions, the free Mg^{2+} and $MgATP$ concentrations are greatly decreased. It therefore appears that a great part of the ATPase activity of these microsomes is due to the Ca^{2+} -independent activity. It should be noted here that, in these (and other) studies, we have noted a large variation in the Mg^{2+} -ATPase activity of the microsomal fraction. We are unable to account for this variability at this time, other than to suggest that this may reflect variation in the endogenous Mg^{2+} content and/or variation in the amount of Mg^{2+} -ATPase present in the isolated microsomal fraction.

Supplementing the CDTA-containing reaction medium with $2.2mM$ - Ca^{2+} ($77\mu M$ free Ca^{2+}) resulted in a 7.5-fold increase in the ATPase activity. This activity is probably due to both the Ca^{2+} -ATPase and Mg^{2+} -ATPase, since under these conditions the free Mg^{2+} and the $MgATP$ concentrations are both elevated to a degree that

Fig. 2. Mg^{2+} -dependence of ^{32}P -labelled hepatic microsomal proteins

Phosphorylation, SDS/polyacrylamide-gel electrophoresis and autoradiography were performed as described in the Experimental section. Additions to the basic incubation medium were: lane 1, none; lane 2, $2mM$ -EGTA; lane 3, $2mM$ -CDTA; lane 4, $2mM$ -CDTA + $1mM$ - $CaCl_2$; lane 5, $2mM$ -CDTA + $1.5mM$ - $MgCl_2$; lane 6, $2mM$ -CDTA + $0.7mM$ - $MgCl_2$ + $1mM$ - $CaCl_2$. The resultant free Ca^{2+} , free Mg^{2+} and $MgATP$ concentrations were (respectively): lane 1, $22\mu M$, $0.5mM$, $0.15\mu M$; lane 2, $11nM$, $0.5mM$, $0.16\mu M$; lane 3, $9nM$, $0.8\mu M$, $1nM$; lane 4, $1.4\mu M$, $2.5\mu M$, $3.2nM$; lane 5, $0.3\mu M$, $93\mu M$, $77nM$; lane 6, $59\mu M$, $0.2mM$, $0.11\mu M$.

Table 3. Effect of CDTA or EGTA on microsomal ATPase activity

ATPase activities were determined as described in the Experimental section, and the values given are the means $\pm$ S.E.M. for three experiments. The species concentrations were calculated as described in the Experimental section.

Addition	[Mg^{2+}] (μM)	[$MgATP$] (mM)	[Ca^{2+}] (μM)	ATPase activity (nmol of P_i /h per mg of protein)
+ $2mM$ -EGTA	34	0.36	0.015	1216 ± 34
+ $2mM$ -CDTA	0.5	0.007	0.011	193 ± 104
+ $2mM$ -CDTA + $2.2mM$ - Ca^{2+}	23	0.22	77.0	1429 ± 136
+ $2mM$ -CDTA + $2mM$ - Mg^{2+}	46	0.46	0.21	1702 ± 56
+ $2mM$ -CDTA + $2mM$ - Mg^{2+} + $0.5mM$ - Ca^{2+}	110	0.81	10.0	1917 ± 90

would produce substantial Mg^{2+} -ATPase activity. When 2 mM- Mg^{2+} is added in place of Ca^{2+} , a 9-fold increase in activity was found. This activity probably reflects predominantly the Mg^{2+} -dependent activity, since under these conditions (less than 0.5 μM free Ca^{2+} , 46 μM free Mg^{2+}) one would expect the Mg^{2+} -ATPase to be very active (see Table 3, '+2 mM-EGTA'). When both Ca^{2+} and Mg^{2+} are present, an even higher activity was found, reflecting both Ca^{2+} -ATPase and Mg^{2+} -ATPase activities.

Thus, on a coarse level at least, a distinction between Ca^{2+} -ATPase and Mg^{2+} -ATPase activities can be made by differential use of EGTA and CDTA. However, the use of CDTA presents problems due to competitive binding between Ca^{2+} and Mg^{2+} . In the presence of CDTA, addition of Ca^{2+} results also in an increase in the free Mg^{2+} concentration, thus making it difficult at best to determine the relative activities of the Ca^{2+} -ATPase and the Mg^{2+} -ATPase.

Discussion

We have presented data describing the phosphorylated intermediates of two hepatic microsomal ATPases. The Ca^{2+} -dependent 125000- M_r phosphoprotein is assumed to be the phosphoenzyme intermediate of the Ca^{2+} -ATPase for the following reasons. First, the phosphoprotein is formed under incubation conditions favourable for enzyme intermediate formation, i.e. short incubation periods (5–10 s), low temperature (0–4°C) and low concentrations of ATP (0.15–0.20 μM). Secondly, the Ca^{2+} -dependent phosphoprotein is labile in hydroxylamine and in NaOH. This is characteristic of an acyl phosphate linkage, as is found in Ca^{2+} -ATPases from other sources (Caroni & Carafoli, 1981; Chan & Junger, 1983; De Jonge *et al.*, 1981; De Smedt *et al.*, 1983; Le Peuch *et al.*, 1983; Tada *et al.*, 1982).

The M_r of the Ca^{2+} -ATPase was estimated from SDS/polyacrylamide-gel electrophoresis of the enzyme intermediate, and was found to be approx. 125000. This is well within the range of 100000–150000 reported for the Ca^{2+} -ATPases from rat hepatic microsomal fraction (Famulski & Carafoli, 1982), dog heart sarcolemma (Caroni & Carafoli, 1981), human erythrocytes (Niggli *et al.*, 1979), rat liver plasma membranes (Chan & Junger, 1983), rat duodenal mucosa plasma membranes (De Jonge *et al.*, 1981), pig plasma membranes (De Smedt *et al.*, 1983), human platelet membranes (Le Peuch *et al.*, 1983) and cardiac sarcoplasmic reticulum (Tada *et al.*, 1982).

The Ca^{2+} -ATPase is specific for Ca^{2+} , as opposed to Mg^{2+} , since free Mg^{2+} concentrations as high as 1 mM were not able to replace the Ca^{2+} .

In the absence of Ca^{2+} , a Ca^{2+} -independent phosphoenzyme intermediate was found. The phosphate linkage of this phosphoprotein was stable in hydroxylamine and therefore probably represents a phosphoester linkage. This type of phosphoprotein is characteristic of the enzyme intermediate of phosphatases (Lazdunski *et al.*, 1971) and of the product of protein kinases (Tada *et al.*, 1982). The 30000- M_r phosphoprotein described here is probably not the product of a protein kinase, for two reasons. First, the incubation conditions (i.e. 0–4°C, 5–10 s, 0.15–0.20 μM -ATP) are not favourable for protein kinases. Second, the ^{32}P -labelled phosphoprotein is rapidly (10 s) abolished when the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ is isotopically diluted with unlabelled ATP (C. R. Fleschner & N. Kraus-Friedmann, unpublished work). Thus the 30000- M_r phosphoprotein is probably not the product of a protein kinase, but the possibility remains that it represents the enzyme intermediate of a phosphatase. Regardless of the exact nature of this activity, it is expressed under the ATPase assay conditions that we use, and therefore will be called an ATPase for the sake of discussion. The exact nature of the Ca^{2+} -independent ATPase activity requires further investigation.

The Ca^{2+} -independent ATPase required Mg^{2+} . In the presence of EGTA, the Ca^{2+} -independent ATPase is active, as evidenced both by enzyme activity and by enzyme-intermediate formation. In the presence of CDTA, however, the Ca^{2+} -independent ATPase activity is greatly decreased, and the formation of an enzyme intermediate is abolished. The addition of Mg^{2+} to the CDTA-containing medium restores both the ATPase activity and the enzyme-intermediate formation. Therefore we have called this activity a Mg^{2+} -ATPase.

The Mg^{2+} -ATPase is apparently responsible for the high basal Mg^{2+} -ATPase activity of hepatic microsomal fraction noted previously by us (Andia-Waltenbaugh *et al.*, 1980) and others (Dawson & Fulton, 1983). The results presented here indicate that this basal Mg^{2+} -ATPase activity may be very effectively decreased by chelating the endogenous Mg^{2+} with CDTA. However, our data indicate that care must be taken when using CDTA, owing to competitive binding between Ca^{2+} and Mg^{2+} .

During revision of this manuscript, a publication has appeared describing a 118000- M_r phosphoprotein as the enzyme intermediate of the hepatic microsomal Ca^{2+} -ATPase (Heilmann *et al.*, 1984).

We thank Ms. Renée Miller for efficient typing of the manuscript. This work was supported by Grant PCM-8114547 from the National Science Foundation.

References

- Andia-Waltenbaugh, A. M. & Friedmann, N. (1978) *Biochem. Biophys. Res. Commun.* **82**, 603–608
- Andia-Waltenbaugh, A. M., Lam, A., Hummel, L. & Friedmann, N. (1980) *Biochim. Biophys. Acta* **630**, 165–175
- Bers, D. M. (1982) *Am. J. Physiol.* **242**, C404–C408
- Bonis, D. & Rossignol, B. (1982) *FEBS Lett.* **137**, 63–66
- Bruns, D. E., McDonald, J. M. & Jarrett, L. (1976) *J. Biol. Chem.* **251**, 7191–7197
- Bygrave, F. L. (1978) *Biochem. J.* **170**, 87–91
- Caroni, P. & Carafoli, E. (1981) *J. Biol. Chem.* **256**, 3263–3270
- Chan, K. M. & Junger, K. D. (1983) *J. Biol. Chem.* **258**, 4404–4410
- Colca, J. R., Kotagal, N., Lacy, P. E. & McDaniel, M. L. (1983) *Biochem. J.* **212**, 113–121
- Dawson, A. P. (1982) *Biochem. J.* **206**, 73–79
- Dawson, A. P. & Fulton, D. V. (1983) *Biochem. J.* **210**, 405–410
- De Jonge, H. R., Ghijsen, W. E. J. M. & Van Os, C. H. (1981) *Biochim. Biophys. Acta* **647**, 140–149
- De Smedt, H., Parys, J. B., Borghraef, R. & Wuytack, F. (1983) *Biochim. Biophys. Acta* **728**, 409–418
- Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75**, 463–505
- Famulski, K. & Carafoli, E. (1982) *Cell Calcium* **3**, 263–281
- Fleschner, C. R., Kraus-Friedmann, N. & Wibert, G. (1984) *Fed. Proc. Soc. Exp. Biol. Fed. Am.* **43**, 1875
- Heilmann, C., Spanner, C. & Gerok, W. (1984) *J. Biol. Chem.* **259**, 11139–11144
- Iwasa, Y., Iwasa, T., Higashi, K., Matsui, K. & Miyamoto, E. (1982) *Biochem. Biophys. Res. Commun.* **105**, 488–494
- Kraus-Friedmann, N., Biber, J., Murer, H. & Carafoli, E. (1982) *Eur. J. Biochem.* **129**, 7–12
- Lanzetta, P. A., Alvarez, L. J., Reinach, P. S. & Candia, O. A. (1979) *Anal. Biochem.* **100**, 95–97
- Lazdunski, M., Petitclerc, C., Chappelet, P. & Lazdunski, C. (1971) *Eur. J. Biochem.* **20**, 124–139
- Le Peuch, C. J., Le Peuch, D. A. M., Katz, S., Demaille, J. G., Hincke, M. T., Bredoux, R., Enouf, J., Levy-Toledano, S. & Caen, J. (1983) *Biochim. Biophys. Acta* **731**, 456–464
- Linnett, P. E. & Beechey, R. B. (1979) *Methods Enzymol.* **55**, 472–518
- Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210
- McDonald, J. M., Bruns, D. E., Jarrett, L. & Davis, J. E. (1977) *Anal. Biochem.* **82**, 485–492
- Moore, L., Fitzpatrick, D. F., Chen, T. S. & Landon, E. J. (1974) *Biochim. Biophys. Acta* **345**, 405–418
- Moore, L., Chen, T., Knapp, H. R. & Landon, E. J. (1975) *J. Biol. Chem.* **250**, 4562–4568
- Moore, P. B. & Kraus-Friedmann, N. (1983) *Biochem. J.* **214**, 69–75
- Niggli, V., Penniston, J. T. & Carafoli, E. (1979) *J. Biol. Chem.* **254**, 9955–9958
- Robinson, J. D. & Lust, W. D. (1968) *Arch. Biochem. Biophys.* **125**, 286–294
- Selinger, Z., Nains, E. & Lasser, M. (1970) *Biochim. Biophys. Acta* **203**, 326–334
- Sillen, L. G. & Martell, A. E. (1971) *Stability Constants of Metal-Ion Complexes: Spec. Publ. Chem. Soc.* **25**
- Tada, M., Yamada, M., Kadoma, M., Inui, M. & Ohmori, F. (1982) *Mol. Cell. Biochem.* **46**, 73–95