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Evidence for the Participation of a Ca^{2+}-Dependent Protein Kinase and Protein Phosphatase in the Regulation of the Ca^{2+} Transport ATPase of the Sarcoplasmic Reticulum. 2. Effect of Phosphorylase Kinase and Phosphorylase Phosphatase†

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ABSTRACT: Dialysis of membranes of the sarcoplasmic reticulum against EDTA solubilizes polypeptides of molecular weight 140 000 and 120 000, corresponding to the α and β subunits of phosphorylase kinase; 100 000, phosphorylase *b* or ATPase; 58 000, calsequestrin; and 31 000, acidic proteins. Fourteen percent of the Ca^{2+}-dependent protein kinase and 13% of the protein phosphatase activity remain associated with these membranes. As a result the oxalate/Ca^{2+} activity ratio of the Ca^{2+} transport ATPase decreases from 1.9 to 1.1. Upon readdition of purified phosphorylase kinase and phosphorylase phosphatase this ratio increases to 1.8. Similarly, the oxalate/Ca^{2+} activity rate is also increased ca. twofold in untreated vesicles. Addition of phosphorylase phosphatase to vesicles isolated under conditions which optimally preserve the Ca^{2+}-dependent protein kinase and protein phosphatase activity reduces the Ca^{2+} transport ATPase activity maximally to 90%. Correspondingly the oxalate/Ca^{2+} activity ratio increases ca. ninefold. Phosphorylase phosphatase is also able to reduce the Ca^{2+} uptake rate by ca. 70% as measured in presence of 0.5 mM oxalate. The ATPase inhibition caused by phosphorylase phosphatase can be specifically reversed by phosphorylase kinase as a function of its concentration. The effect of phosphorylase is a function of the age of the vesicles. During 15 days the ATPase is desensitized and only minimally responds to phosphorylase phosphatase addition. The amount of incorporated phosphate can be reduced by antiphosphorylase kinase by 59% and by phosphorylase phosphatase by 39%. Phosphorylase kinase alone increases the degree of phosphorylation by 7%.

It has been shown that an inhibition of a Ca^{2+}-dependent protein kinase which is present in purified sarcoplasmic reticulum from rabbit skeletal muscle either with anti-phosphorylase kinase or with hydroxylamine leads to a concomitant inhibition of the Ca^{2+} transport ATPase (Hörl et al., 1975a, 1978; Hörl and Heilmeyer, 1976a,b; Gröschel-Stewart et al., 1976). The Ca^{2+} uptake rate and maximal Ca^{2+} accumulation are reduced parallel to a decrease of the Ca^{2+} transport ATPase activity caused by anti-phosphorylase kinase or phosphorylase *b* (Hörl et al., 1975b, 1978). Oxalate or fluoride stimulates the Ca^{2+} transport ATPase activity which can be correlated with an inhibition of the protein phosphatase. The antagonistic effects of hydroxylamine and oxalate on the ATPase activity suggest that the ATPase activity is regulated by the activity ratio of a Ca^{2+}-dependent protein kinase and protein phosphatase (Hörl et al., 1978).

A protein kinase which is activated by Ca^{2+} concentrations between 10^{-8} and 10^{-5} M and which accepts several proteins as substrate is phosphorylase kinase (Heilmeyer et al., 1970; Brostrom et al., 1971; Kilimann and Heilmeyer, 1977); a general protein phosphatase seems to be phosphorylase phosphatase (Antoniw and Cohen, 1975; Djovkar and Heilmeyer, 1978). Since the former enzyme is available as homogeneous protein and the latter as highly enriched preparation, it seemed

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TABLE I: Solubilization of Enzymes from Sarcoplasmic Reticulum Membranes by EDTA Treatment.[a]

Fraction	Vol (mL)	Protein (mg/mL)	ATPase U/mg	ATPase Units	ATPase %	Ca^{2+}-dependent protein kinase U/mg	Ca^{2+}-dependent protein kinase Units	Ca^{2+}-dependent protein kinase %	Protein phosphatase U/mg	Protein phosphatase Units	Protein phosphatase %	Phosphorylase U/mg	Phosphorylase Units	Phosphorylase %
Untreated vesicles	1	16.10	0.813	13.1	100	4.2	67.6	100	0.25	4.03	100	4.9	78.9	100
EDTA-treated vesicles	1.8	12.54	3.294	74.4	568	0.43	9.7	14.3	0.023	0.52	12.9	Not detectable		
Supernatant	2.5	0.99	0.847	2.1	16.0	13.2	32.7	48.4	1.6	4.0	99.3	19.9	49.8	63.1

[a] One milliliter suspension of vesicular protein was dialyzed against 300 mL of 10 mM EDTA, pH 9.0, for 15 h at 4 °C. The content of the dialysis bag was centrifuged for 15 min at 11 000*g*. The pellet was resuspended in 1.5 mL of 0.1 M NaCl and recentrifuged as before. The two supernatants were combined and dialyzed against 1 L of 0.1 M NaCl for 12 h at 4 °C. The pellet was washed as before with 20 mL of 0.1 M NaCl and resuspended in 1.5 mL of 0.1 M NaCl. The enzymatic activities were determined as described in Experimental Procedure.

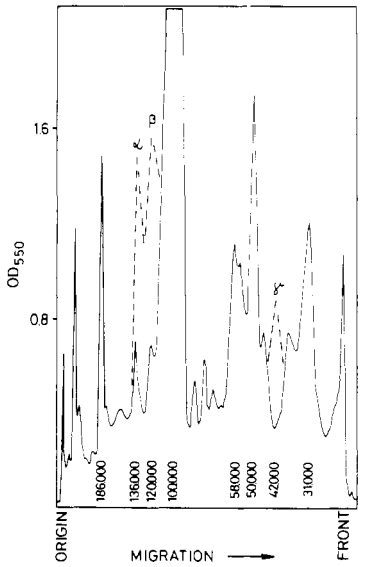

FIGURE 1: Polyacrylamide gel electrophoresis of purified vesicles of the sarcoplasmic reticulum. Purified sarcoplasmic reticulum was denatured with $NaDodSO_4$ and subjected to polyacrylamide gel electrophoresis as described in Experimental Procedure. To 68 μg of vesicular protein was added 16 μg of purified phosphorylase kinase and the mixture was treated as described above. The densitometric tracings of both gels were superimposed. The difference showing the subunits of purified phosphorylase kinase is indicated by the dotted line.

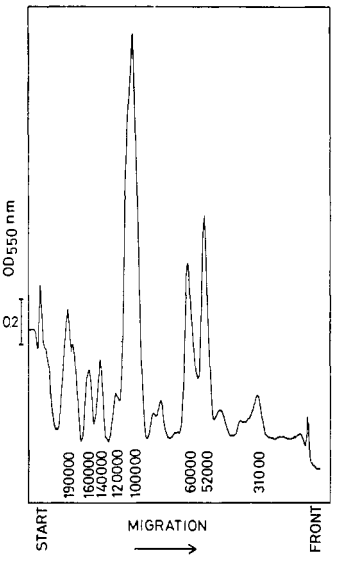

FIGURE 2: Solublized polypeptides by EDTA treatment from membranes of the sarcoplasmic reticulum. Twenty-eight micrograms of protein of the supernatant of EDTA treated vesicles (see Table I) was separated by polyacrylamide gel electrophoresis as described in Experimental Procedures.

feasible to study directly the influence of these two regulatory enzymes on the Ca^{2+} transport ATPase. This publication will show that the protein phosphatase concomitantly diminishes the Ca^{2+} transport ATPase activity, the steady-state level of the incorporated phosphate and the Ca^{2+} uptake rate which is reversed by the Ca^{2+}-dependent protein kinase.

Experimental Procedure

Unless otherwise stated all Methods and Materials are the same as described by Hörl et al. (1978).

Results

Besides the Ca^{2+} transport ATPase (mol wt 100 000, ca. 75% of the total protein) several other polypeptides are present in the sarcoplasmic reticulum. Two of them with apparent molecular weights of 140 000 and 120 000 electrophoretically comigrate as $NaDodSO_4$[1] complexes with the α and β subunits of phosphorylase kinase. The γ subunit of the added phosphorylase kinase can be detected as a new polypeptide band (Figure 1). By dialysis of the vesicles against EDTA and resedimentation, the membranes are freed of the contaminating phosphorylase *b* activity and 63% activity is recovered in the supernatant (see Table I). In contrast ca. 14% of the Ca^{2+}-dependent protein kinase and ca. 13% of the protein phosphatase activity remain associated with these membranes. The specific activity of the Ca^{2+} transport ATPase rises nearly fourfold in the membrane fraction. A low amount (ca. 16%) of ATPase activity of similar specific activity as the original Ca^{2+} transport ATPase is detectable in the supernatant. Since 25% of the solubilized protein represents phosphorylase *b* (calculated employing a specific activity of 80 U/mg), the ATPase activity in the supernatant can partially be produced by the combined action of phosphorylase kinase and phosphatase on phosphorylase. One of the solubilized polypeptides shows an apparent molecular weight of ca. 100 000; it represents mainly phosphorylase *b* (see Figure 2). In addition polypeptides of molecular weight of ca. 140 000 and 120 000, corresponding to the α and β subunits of phosphorylase kinase, are solubilized. A protein of molecular weight 31 000 which may represent the protein phosphatase (Gratecos et al., 1974) is also detectable in the supernatant. Other major solubilized polypeptides have apparent molecular weights in the range of calsequestrin (Mac Lennan and Holland, 1975). The sedimented vesicles still contain a 100 000 molecular weight component, the ATPase, calsequestrin, and the 31 000 molecular weight polypeptide (see Figure 3). The EDTA treated vesicles can no longer accumulate Ca^{2+} (not shown) and the oxalate/Ca^{2+} activity ratio of the Ca^{2+} transport ATPase is

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol.

TABLE II: Partial Reconstitution of the Oxalate Stimulation of the Ca^{2+} Transport ATPase by Phosphorylase Kinase and Phosphorylase Phosphatase.[a]

Preparation	Conditions			
	ATP (5 mM)	Free calcium (1.4×10^{-6} M)	Oxalate (4 mM)	Oxalate/calcium act. ratio
Untreated vesicles	0.104	0.608	1.169	1.92
EDTA-treated vesicles (15 h)	0.827	4.530	4.960	1.09
Addition				
Phosphorylase phosphatase (30 μg/mL), phosphorylase kinase (10 μg/mL), untreated vesicles	0.074	0.393	1.503	3.82
Phosphorylase phosphatase (30 μg/mL), phosphorylase kinase (10 μg/mL), EDTA-treated vesicles (15 h)	0.662	3.416	6.116	1.79

[a] To 0.1 mg/mL vesicular protein phosphorylase phosphatase (0.045 U/mg) and phosphorylase kinase (7.35 U/mg) were added as indicated. The incubation mixture corresponded to that for the Ca^{2+} transport ATPase assay. The ATPase activities were determined as described in Experimental Procedure and were expressed as μmol min^{-1} mg^{-1}.

TABLE III: Effect of Phosphorylase Phosphatase and Phosphorylase Kinase on the Activity of the Ca^{2+} Transport ATPase.[a]

Addition	ATP (5 mM)	Free calcium (1.4×10^{-6} M)	Oxalate (4 mM)	Oxalate/calcium ratio
	0.205	0.528	1.00	1.89
Phosphorylase phosphatase (53 μg/mL)	0.185	0.183	0.895	4.89
Phosphorylase phosphatase (106 μg/mL)	0.180	0.066	0.847	12.80
Phosphorylase phosphatase (53 μg/mL), phosphorylase kinase (24 μg/mL)	0.215	0.277	0.845	3.05
Phosphorylase phosphatase (106 μg/mL), phosphorylase kinase (24 μg/mL)	0.206	0.286	0.799	2.79
Phosphorylase kinase (24 μg/mL)	0.195	0.493	0.946	1.92

[a] Vesicles of the sarcoplasmic reticulum were isolated as described in the text. To 0.1 mg/mL vesicular protein, phosphorylase phosphatase (0.045 U/mg) and phosphorylase kinase (7.35 U/mg) were added as indicated. The incubation mixture corresponded to that of the Ca^{2+} transport ATPase assay. The ATPase activities were determined as described in Experimental Procedure and were expressed as μmol min^{-1} mg^{-1}.

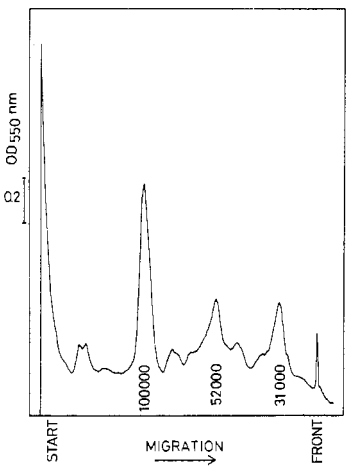

FIGURE 3: Polypeptides of the sarcoplasmic reticulum following EDTA treatment. Twenty-five micrograms of protein of the EDTA treated vesicles (see Table I) was separated by polyacrylamide gel electrophoresis as described in Experimental Procedure.

reduced from 1.9 to 1.1 (Table II). This ratio increases again to 1.8 by addition of phosphorylase kinase and phosphorylase phosphatase (Table II). In order to optimally preserve the Ca^{2+}-dependent protein kinase and protein phosphatase activity during purification of the vesicular fraction conditions were chosen which were similar to those employed for the isolation of these two regulatory enzymes: EGTA was used instead of EDTA in order to prevent extraction of Mn^{2+} which activates the protein phosphatase (Djovkar and Heilmeyer, 1978). Since phosphorylase kinase is inhibited by oxidation of SH groups (Hayakawa et al., 1973; Cohen, 1973) 1 mM DTE was included in the extraction buffer. Phosphorylase phosphatase is very unstable at a pH value lower than 5.6 (Djovkar and Hielmeyer, 1978); therefore at each step the pH was adjusted to 7.0. Under these conditions mainly the specific activity of the protein phosphatase in the crude extract was increased ca. tenfold but in the vesicular fraction the specific activity was 0.21 U/mg as found in the earlier preparation. The specific activity of the membrane bound protein kinase assayed with the substrate phosphorylase *b* was diminished ca. fourfold; phosphorylase *b* was nearly completely absent (0.01 U/mg). Probably due to the change of the kinase/phosphatase activity ratio, the specific activity of the Ca^{2+} transport ATPase activity was reduced by ca. 20%.

Upon addition of increasing amounts of the protein phosphatase to this preparation only a minor reduction (ca. 10%) of the basal ATPase activity is observed (Table III), whereas the Ca^{2+} stimulated ATPase activity can be reduced maximally to ca. 90%. This effect can be partially reversed by addition of the antagonistic enzyme phosphorylase kinase; ca. 55% of the original ATPase activity is regained. The same amount of phosphorylase kinase alone causes only a negligible effect on the Ca^{2+} transport ATPase activity. Oxalate maximally stimulates the Ca^{2+} transport ATPase and only weak reductions (10–20%) by the two added enzymes are observed. Therefore, the oxalate/Ca^{2+} activity ratio of the ATPase increases upon phosphorylase phosphatase addition as a function of its concentration ca. ninefold and decreases again following kinase addition to nearly the ratio measured without addition

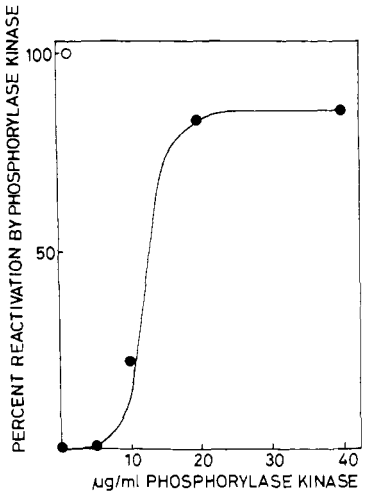

FIGURE 4: Reversal of the phosphorylase phosphatase induced Ca^{2+} transport ATPase inhibition by phosphorylase kinase. To 0.1 mg/mL vesicular protein (specific activity of the Ca^{2+} transport ATPase in absence of oxalate: 0.74 μmol min^{-1} mg^{-1}), 30 μg/mL phosphorylase phosphatase (0.045 U/mg) was added. The incubation mixture corresponded to that of the Ca^{2+} transport ATPase assay and contained additionally 10^{-5} M Mn^{2+}. One hundred percent activity represents the ATPase activity without addition (○), 0 represents the ATPase activity obtained in presence of phosphorylase phosphatase (0.42 μmol min^{-1} mg^{-1}). Phosphorylase kinase was added in the amounts as indicated. Ca^{2+} transport ATPase activity in absence of oxalate was determined as described in Experimental Procedure.

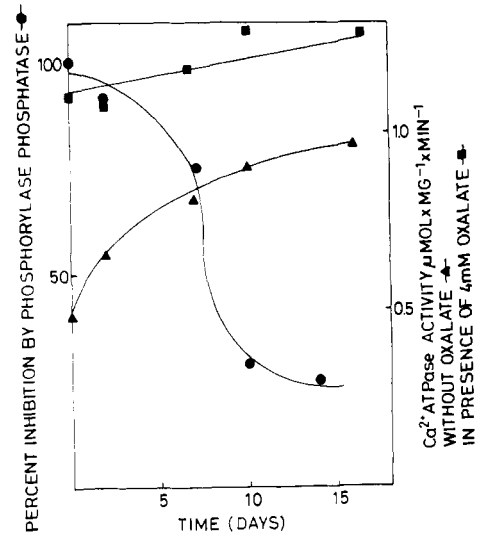

FIGURE 5: Effect of aging of the sarcoplasmic reticulum. To 0.1 mg/mL vesicular protein, 30 μg of phosphorylase phosphatase (0.045 U/mg) was added. The difference of the Ca^{2+} transport ATPase activities in absence (0.50 μmol min^{-1} mg^{-1}) and presence of phosphorylase phosphatase (0.34 μmol min^{-1} mg^{-1}) represents 100% inhibition at zero time. The same difference measured at the later time points is expressed as percent of the activity difference of the first day. Other ATPase activities are assayed as described in Experimental Procedure.

of these two enzymes. As control phosphorylase phosphatase was denatured by boiling; this material has no effect on the ATPase activities.

The inhibition of the ATPase caused by phosphorylase phosphatase is reversed to ca. 80% by increasing phosphorylase kinase concentrations (Figure 4).

Storage of the vesicles at 0 °C for 15 days abolishes nearly completely the inhibitory effect of phosphorylase phosphatase (Figure 5). In the same time period the Ca^{2+} transport activity assayed in absence of oxalate increases ca. twofold whereas it is nearly unchanged when determined in presence of oxalate (ca. 10% increase). Therefore the oxalate/Ca^{2+} activity ratio decreases from 2.3 to 1.3 during this time period. The maximal Ca^{2+} uptake capacity does not change considerably (not shown).

Concomitantly with a ca. 75% decrease of the Ca^{2+} transport ATPase activity by phosphorylase phosphatase the Ca^{2+} uptake rate in presence of 0.05 mM oxalate is also reduced (Figure 6). The degree of this reduction (ca. 68% and 71%) differs only slightly at the two free Ca^{2+} concentrations of 2.6×10^{-7} and 1.7×10^{-6} M, respectively. The influence of phosphorylase kinase on the Ca^{2+} uptake rate is not shown because the Ca^{2+} binding properties of this enzyme (Bronstrom et al., 1971; Kilimann and Heilmeyer, 1977) interfere with the test under these low Ca^{2+} uptake rates (Makinose, 1975).

An anti-phosphorylase kinase concentration which inhibits the Ca^{2+} transport ATPase activity to 50–60% (compare Figure 3 in Hörl et al., 1978) reduces the level of the incorporated phosphate by ca. 59% (Table IV). Similarly, the protein phosphatase is also able to reduce this incorporated amount by ca. 40%, whereas addition of phosphorylase kinase alone causes only a minor increase.

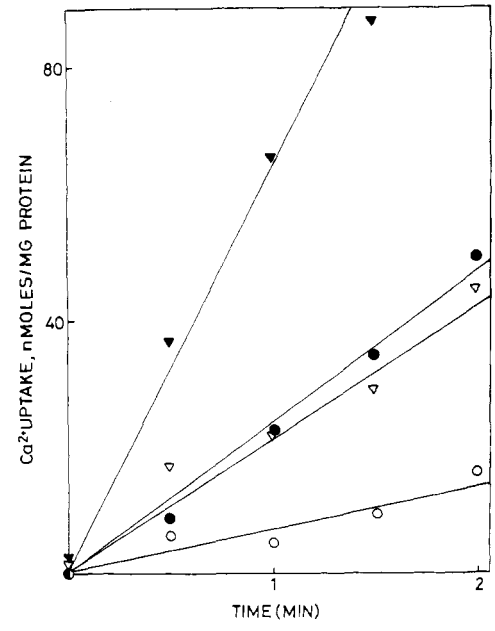

FIGURE 6: Inhibition of Ca^{2+} uptake of the sarcoplasmic reticulum by phosphorylase phosphatase. The Ca^{2+} uptake of the sarcoplasmic reticulum was assayed as described in Experimental Procedure at 1.7×10^{-6} M (▼) and 2.6×10^{-7} M (●) free Ca^{2+}. The incubation mixture corresponds to that of the Ca^{2+} transport ATPase assay except that 0.5 mM oxalate was employed. In addition the incubation mixture at both free Ca^{2+} concentrations contained 0.55 mg/mL phosphorylase phosphatase (0.045 U/mg) (○ and ▽). The reaction was started by addition of ATP and immediately a sample was removed (ca. 3 s) and filtered through a Millipore filter. The amount of Ca^{2+} adsorbed measured at this time was subtracted from the amount accumulated at the later time points.

Discussion

The hypothesis that a Ca^{2+}-dependent protein kinase activates and antagonistically a protein phosphatase inhibits the Ca^{2+} transport ATPase is suggested by the presence of both enzymes in purified sarcoplasmic reticulum of rabbit skeletal muscle (compare Hörl et al., 1978). In $NaDodSO_4$ gels polypeptides corresponding in molecular weight to the subunits, α and β but not γ, of phosphorylase kinase can be detected (compare Figure 1). This latter polypeptide might be com-

TABLE IV: Effect of Phosphorylase Kinase, Anti-Phosphorylase Kinase and Phosphorylase Phosphatase on the Phosphate Incorporation of the Sarcoplasmic Reticulum.[a]

	mol of phosphate/10^5 g of protein	%
Control vesicles (1.5 mg/mL)	0.368	100
Addition		
Phosphorylase kinase (0.3 mg/mL)	0.395	107.3
Anti-phosphorylase kinase (4.5 mg/mL)	0.151	41.0
Phosphorylase phosphatase (0.5 mg/mL)	0.223	60.6

[a] The amount of phosphate incorporated was determined as described in Methods. However, the ATP regenerating system was omitted. Phosphorylase kinase (7.35 U/mg), anti-phosphorylase kinase (purified γ-globulin fraction) (see Hörl et al., 1978) and phosphorylase phosphatase (0.045 U/mg) were added as indicated.

pletely absent or present below stoichiometric amounts. An exchange of the α and β subunits vs. γ must occur in the cell since the γ subunit is synthesized at a ca. twofold slower rate than the α and β subunits (Jennissen et al., 1974, 1976). Therefore another membrane component might replace the γ subunit. However, none of the polypeptides with molecular weights of 100 000 or 50 000–54 000 or 30 000–34 000 seem to show immunological similarity to any of the subunits of phosphorylase kinase since none of these components cross-react in the $NaDodSO_4$ denatured form with either anti-α or anti-β or anti-γ (Hörl et al., 1978).

From the $NaDodSO_4$ gel pattern of the vesicular polypeptides one can estimate that approximately 10 μg of α and β per mg of vesicular protein are present. Calculating with a specific activity of 8.0 μmol min^{-1} mg^{-1} for the soluble phosphorylase kinase ca. 1 μg of phosphorylase kinase should be present in 1 mg of vesicular protein. This difference might be due to a lower specific activity of the membrane bound enzyme when phosphorylase *b* as a nonphysiological substrate is used. In agreement with this interpretation glycogen phosphorylase seems not to be associated with membrane fractions but rather with glycogen (Meyer et al., 1970). Treatment of the membranes with EDTA, which solubilizes loosely associated proteins (Duggan and Martonosi, 1970) leads to a decrease of the specific activity of the Ca^{2+}-dependent protein kinase and of the protein phosphatase (see Table I). In agreement, polypeptides of molecular weights 140 000 and 120 000, corresponding to those of the α and β subunits of phosphorylase kinase, are found in the supernatant whereas the treated membranes contain nondetectable amounts (compare Figures 1, 2, and 3). The residual 14% of the Ca^{2+}-dependent protein kinase and protein phosphatase activities which are still associated with the membranes might not be detectable in the $NaDodSO_4$ gel electrophoresis. The loss of most of the Ca^{2+}-dependent protein kinase and protein phosphatase decreases the oxalate/Ca^{2+} activity ratio from 1.9 to 1.1. The concomitant increase of the Ca^{2+} transport ATPase activity indicates that the enzyme may be desensitized (compare Table II). The EDTA treatment causes also an increase of the basal ATPase activity (see Table II). This ATP hydrolysis is probably catalyzed by the same enzyme (Froehlich and Taylor, 1976). It indicates a further step in the desensitization process. This desensitization may be influenced by other proteins with molecular weights of 52 000–58 000 in the range of calsequestrin or 31 000 which are released from the membranes by EDTA. However, this desensitization can be partially reversed by addition of phosphorylase kinase and phosphorylase phosphatase.

Another kind of desensitization may occur with aging since such preparations respond very little to the addition of the protein phosphatase (Figure 5). Parallel to this desensitization the activity of the Ca^{2+} stimulated transport ATPase increases ca. twofold and reaches nearly the same activity as determined in presence of oxalate. This latter activity increases during this time period only ca. 10%. It suggests that in presence of oxalate the maximal activity without the influence of the phosphatase is observed. The desensitization may be due to a partial proteolytic degradation similar to that observed in the transition of phosphorylase *b* to *b'*, i.e., a loss of phosphorylatable peptide (Fischer et al., 1959). Untreated membranes respond upon addition of protein phosphatase as a function of its concentration in absence but not in presence of oxalate with reduction of the Ca^{2+}-stimulated ATPase activity maximally by ca. 90% (Table III). As a consequence, the oxalate/Ca^{2+} activity ratio increases ca. ninefold. If there is no indirect way by which this phosphatase inhibits the Ca^{2+} transport ATPase activity (for example, by closing the Ca^{2+} transport channels which then are reopened by oxalate), it suggests that this enzyme dephosphorylates the Ca^{2+} transport ATPase. In agreement, the protein phosphatase reduces the level of the covalently bound phosphate by ca. 40% (see Table IV). Parallel to this the Ca^{2+} uptake rate is also diminished by addition of the protein phosphatase.

The inhibition caused by the phosphatase can be reversed specifically with phosphorylase kinase as a function of its concentration which may be interpreted as an acceleration of a phosphorylation reaction. It seems that freshly isolated vesicles contain sufficient Ca^{2+}-dependent protein kinase activity to maximally activate the Ca^{2+} transport ATPase since addition of phosphorylase kinase alone does not significantly enhance the Ca^{2+} transport ATPase activity or the phosphoprotein level (compare Tables III and IV). It was reported by Schwartz et al. (1976) that phosphorylase kinase does not invariably activate the Ca^{2+} uptake rate of fast twitch skeletal sarcoplasmic reticulum.

The balance of the Ca^{2+}-dependent protein kinase and protein phosphatase activity can also be shifted in favor of the phosphatase by inhibition of the kinase with anti-phosphorylase kinase which again results in a decrease of the Ca^{2+} transport ATPase activity as well as of the phosphoprotein level. It demonstrates that the kinase–phosphatase activity ratio determines the Ca^{2+} transport ATPase activity.

It has been shown by several groups that the major polypeptide which is phosphorylated in rabbit muscle sarcoplasmic reticulum has an apparent molecular weight of ca. 100 000 (Yamamoto and Tonomura, 1967; Martonosi, 1969; Mac Lennan, 1970; Ikemoto et al., 1971; Hasselbach, 1972; Hasselbach et al., 1973). Excess of protein phosphatase or inhibition of the kinase by anti-phosphorylase kinase (compare Table III) or hydroxylamine (see Hörl et al., 1978) leads to a reduction of the phosphoprotein level. This observation suggests that this polypeptide is phosphorylated and dephosphorylated by the Ca^{2+}-dependent protein kinase and protein phosphatase, respectively. The inability of the native phosphoprotein to react with hydroxylamine (Hörl et al., 1978; Inesi et al., 1970) or hydrazine (Makinose, 1969) seems to support this view since the protein kinase incorporates phosphate as phosphoserine or phosphothreonine. However, the identification of an aspartyl phosphate in the denatured protein (Post and Kume, 1973) argues against the possibility that the protein bound phosphate represents only phosphoserine or -threonine. More important

even a higher phosphoprotein level can be obtained by using acetyl phosphate as phosphorylating agent (Friedman and Makinose, 1970). Acetyl phosphate is not accepted by phosphorylase kinase as substrate (Heilmeyer, unpublished). Therefore, a phosphoprotein obtained by the Ca^{2+}-dependent protein kinase reaction cannot be identical with that formed during the catalytic cycle of the ATPase (Yamada et al., 1971; Friedman and Makinose, 1970). The present results do not prove whether the incorporated phosphate is bound exclusively in an acyl or ester linkage; it might be that it is a mixture of both kinds of phosphates.

If phosphorylation regulates the Ca^{2+} transport ATPase minimally 1 mol of phosphate/mol of protein should be incorporated in analogy to other known examples for protein phosphorylation (phosphorylase *b* (Krebs et al., 1958), glycogen synthetase (Larner et al., 1974; Soderling et al., 1970; Cohen et al., 1976), phosphorylase kinase (Hayakawa et al., 1973a,b; Cohen, 1973; Hörl et al., 1975b), troponin (Stull et al., 1972; Pires et al., 1974; Pratje and Heilmeyer, 1974), and myosin light chain (Perry et al., 1975). There are several possibilities why this amount is not found. For example, the isolated enzyme may be partially desensitized as has been shown here to occur with aging. Furthermore, the enzyme may be composed of regulatory and catalytic subunits both having a molecular weight of 100 000 of which only the regulatory subunit is phosphorylated as observed with the cAMP-dependent protein kinase from cardiac muscle (Rangel-Aldao and Rosen, 1976).

In summary, the effects on the Ca^{2+} transport ATPase by the Ca^{2+}-dependent protein kinase and phosphatase lead to the hypothesis that the Ca^{2+} transport ATPase is phosphorylated and dephosphorylated by these two regulatory enzymes. In addition phosphate is incorporated as an intermediate of the catalytic cycle. The reported experiments strongly support this hypothesis but there is not yet final evidence.

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Nuclear Magnetic Resonance Studies on the Binding of Substrate, Coenzymes, and Effectors to Glutamate Dehydrogenase†

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ABSTRACT: The binding of substrate, coenzymes, and effectors to the enzyme glutamate dehydrogenase was studied by ligand nuclear magnetic resonance (NMR) line broadening. Binary complexes, in fast exchange with the free ligand, of the enzyme with the substrate α-ketoglutarate and oxidized and reduced coenzyme were detected by this method. The binding of the inhibitor succinate is comparable to that of the oxidized substrate indicating that neither the precise length of the carbon chain nor the carbonyl group is crucial for the binding. The affinity of the enzyme for the reduced substrate, L-glutamic acid, is apparently much weaker. The formation of ternary complexes with enzyme, substrate, and coenzyme results in a drastic decrease of the observed NMR line broadenings. Quantitative studies show that substrate and coenzyme enhance each others binding by one or more orders of magnitude. Temperature-dependent measurements lead to the proposition that the binding of α-ketoglutarate and oxidized coenzyme in the ternary complex occurs via a first fast step, which is followed by a slow isomerization of the complex. The same two-step binding mechanism seems to occur on formation of the binary complex with reduced coenzyme, but not with oxidized coenzyme. The effects of the allosteric effectors GTP and ADP on the ternary complex formation indicate that inhibition is related to stronger binding and slower release of the bound ligands from the enzyme.

The enzyme glutamate dehydrogenase (L-glutamate: $NAD(P)^+$ oxidoreductase (deaminating), EC 1.4.1.3) from bovine liver catalyzes the reversible oxidation of L-glutamate by NAD(P) to α-ketoglutarate, ammonia, and NAP(P)H. The enzymatic activity is subject to regulatory effects of a large number of compounds of which the inhibitor GTP and the activator ADP are the most studied ones. Despite the large number of studies concerning the enzyme many important questions about the relationship of catalytic and regulatory sites and the role of the subunit structure are still awaiting their final answers.

We have undertaken NMR[1] studies of ligand binding to GluDH which were directed mainly to α-KG and oxidized coenzyme since these compounds are difficult to study by optical techniques. A preliminary report of part of our studies has been published (Andree & Zantema, 1974).

Theory

The theory for nuclear magnetic relaxation of small ligands bound to macromolecules has been well developed in the past years (Swift & Connick, 1962; Navon et al., 1970; Sykes et al., 1970; Beard & Schmidt, 1973). We will briefly review and expand the present theory as far as it is used to interpret our experimental data.

For the equilibrium

$$E + L \rightleftharpoons EL \qquad (1)$$

the following relation between the NMR line width and the total concentrations of ligand (L_0) and enzyme (E_0) holds, provided that the ligand is in large excess over the enzyme:

$$E_0/B = \pi(K_d + L_0)T_2^i \qquad (2)$$

K_d is the dissociation constant for equilibrium 1. B denotes the line broadening of the ligand and specifically means the dif-

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[1] Abbreviations used: NMR, nuclear magnetic resonance; GluDH, glutamate dehydrogenase; α-KG, α-ketoglutarate.

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