PURIFICATION OF (Ca^{2+} + Mg^{2+})-ATPase FROM SMOOTH MUSCLE BY CALMODULIN AFFINITY CHROMATOGRAPHY

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1. Introduction

An ATP-dependent Ca^{2+} accumulation system and a corresponding ATPase activity have been demonstrated in microsomal fractions from smooth muscle [1,2]. Because both the CaMgATPase and the Ca^{2+}-transport were stimulated by calmodulin [3], a purification of the CaMgATPase by calmodulin affinity chromatography was attempted. A similar approach had proven succesfull for the CaMgATPase of human erythrocytes [4,5]. We now report the purification of a calmodulin-sensitive CaMgATPase from smooth muscle of the pig stomach (antral part) up to specific activities comparable to the purified enzyme of human erythrocytes. This purified CaMgATPase does not present a basal Ca^{2+}-independent MgATPase. A $M_r \sim 140\,000$ is estimated from SDS–polyacrylamide gel electrophoresis, a value comparable to that found for MgATPase in human erythrocytes [5,6].

2. Methods

2.1. *Preparation of the microsomes*

Microsomes were prepared from pig antrum smooth muscle [7]. This preparation procedure includes a KCl wash cycle, in order to remove extrinsic proteins.

2.2. *Solubilization of the ATPase*

KCl-extracted microsomes were centrifuged in a Beckman Ti 75 rotor at 50 000 rev./min (223 800 × g_{max}) for 30 min, and resuspended in 130 mM KCl, 20 mM Hepes (pH 7.4), 0.5 mM $MgCl_2$, 0.05 mM $CaCl_2$, 2 mM dithiothreitol at 8 mg protein/ml. An equal volume of 8 mg Triton X-100/ml in the same buffer was added and the mixture stirred for 10 min at 4°C. To remove the unsolubilized material this mixture was centrifuged in the Ti 75 rotor at 50 000 rev./min for 30 min and the pellet was discarded.

2.3. *Calmodulin-Sepharose affinity chromatography*

The Triton X-100-solubilized material (usually ~20 ml), was added to 3 ml of a calmodulin–Sepharose 4B affinity gel that had been equilibrated with buffer A (130 mM KCl, 20 mM Hepes (pH 7.4), 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 2 mM dithiothreitol, 0.05% (w/v) asolectin and 0.4% (w/v) Triton X-100). This mixture was incubated for 1 h at 4°C in an end-over-end mixer. The gel was transferred into a column (1.5 × 1.6 cm) and the effluent of the column collected and pooled. The unbound material was then washed from the column with an additional 10 ml buffer A at 1.4 ml/min.

Thereupon the column was washed with Ca^{2+}-free buffer B (buffer A, but with 2 mM EDTA instead of 0.1 mM $CaCl_2$) at 1.4 ml/min and 1.2 ml fractions were collected. The buffers containing asolectin (A,B) were sonicated under nitrogen in a MSE tip sonicator until they were no longer turbid.

2.4. *ATPase activity and protein*

ATPases were measured at 37°C in a coupled enzyme assay [2,7]. The CaMgATPase, was determined after addition of Ca^{2+} (10 μM) (EGTA-buffered) and the calmodulin was used at 10 μg/ml. Protein was measured after precipitation in a deoxycholate–trichloroacetic acid solution [8] with bovine serum albumin as standard.

Abbreviations: CaMgATPase, (Ca^{2+} + Mg^{2+})-ATPase; NaKATPase, (Na^+ + K^+)-ATPase; Hepes, 4-(2-hydroxyethyl)-1-piperazine–ethane sulphonic acid

2.5. *Electrophoresis*

SDS–polyacrylamide gel electrophoresis was done in a discontinuous buffer system [9], with a stacking gel of 3% and a separating gel of 10% (w/v) acrylamide. The diluted samples of purified CaMgATPase were concentrated before electrophoresis by precipitation in a deoxycholate–trichloroacetic acid solution as described for the protein assay. Boehringer Combithek calibration proteins were used as molecular mass standards.

2.6. *Demonstration of the phosphorylated intermediate*

Phosphorylation was induced at 0°C for 20 s in 1.25 ml medium containing: 100 mM KCl, 30 mM imidazole–HCl (pH 6.8), 0.2 mM EGTA, 0.8 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 μg purified ATPase. The reaction was started by the addition of 0.1 mM [γ-^{32}P]ATP and stopped by adding an equal volume of stop solution: 10% trichloroacetic acid, 50 mM phosphoric acid, 0.5 mM ATP. Bovine serum albumin (2 mg) was added and thereupon the tubes centrifuged, the pellets dissolved and electrophoresed as in [10]. Gels were sliced and the ^{32}P content of the slices was determined. In control experiments $CaCl_2$ was omitted.

2.7. *Materials*

Triton X-100 was from Merck and asolectin from Paesel GmbH (Frankfurt). Calmodulin was prepared from bovine brain [11]. A calmodulin–Sepharose 4B gel was prepared from 1.5 g cyanogenbromide-activated Sepharose and 11 mg calmodulin as in [4].

3. Results and discussion

3.1. *The specific acitivity of the ATPase*

Table 1 summarizes the changes in specific activity of the different ATPases throughout the purification procedure. In the first 2 lines the activities of the KCl-extracted microsomes and of the material solubilized by Triton X-100 are given respectively. Line 3 shows the ATPase activities in the Triton X-100 solubilizate after 1 h equilibration with the calmodulin gel. After washing the gel column free of all non-bound material, the bound CaMgATPase was released by lowering the free [Ca^{2+}]. Line 4 of table 1 shows the ATPase activities in the tube containing the peak activity of CaMgATPase. These findings support the following conclusions:

(1) The KCl-extracted microsomes show a CaMgATPase activity which is stimulated by calmodulin by a factor of 1.6 and also contain a considerable MgATPase activity of unknown origin and some NaKATPase activity;

(2) In the Triton X-100-solubilized fractions, the specific activity of the Mg- and NaKATPase remain

Table 1

Specific activities of the ATPases during the purification procedure (spec. act.; μmol . mg protein^{-1} . min^{-1})

	MgATPase	NaKATPase	CaMgATPase 0 calmodulin	CaMgATPase 10 μg/ml calmodulin	Mean stimulation of CaMgATPase by calmodulin
KCl-extracted microsomes	0.139 ± 0.026 (5)	0.016 ± 0.004 (5)	0.041 ± 0.011 (5)	0.056 ± 0.011 (5)	1.63 ± 0.12 (5)
Triton X-100-solubilized fraction	0.133 ± 0.013 (5)	0.035 ± 0.016 (5)	0.160 ± 0.045 (5)	0.222 ± 0.033 (5)	1.32 ± 0.11 (5)
Fraction not bound to calmodulin gel	0.143 ± 0.031 (5)	0.039 ± 0.007 (5)	0.102 ± 0.030 (5)	0.12 ± 0.025 (5)	1.76 ± 0.40 (5)
Peak eluted with Ca^{2+}-free buffer	0.030 ± 0.0025 (5)	0.014 ± 0.014 (5)	4.12 ± 0.50 (5)	5.70 ± 0.71 (5)	1.41 ± 0.06 (5)
enrichment compared to KCl extracted microsomes	–	–	100 ×	102 ×	
enrichment compared to crude microsomes (7)	–	–	338 ×	384 ×	

– Values are mean ± SEM; The number of observations is given in parentheses

largely unchanged compared to the KCl-extracted microsomes, but that of the CaMgATPase is increased (the CaMgATPase still responds to calmodulin under these conditions).

(3) After incubating the solubilized fraction with the affinity gel in the presence of Ca^{2+}, some CaMgATPase is bound but a considerable part stays in solution, as is indicated by the high specific activity of this enzyme in the unbound material. Moreover the CaMgATPase in the unbound enzyme is stimulated by calmodulin to the same extent as in the Triton X-100 solubilizate.

(4) The CaMgATPase which is bound to the calmodulin affinity gel and which is again released in Ca^{2+}-free medium has a high specific activity of 4.1 and 5.7 μmol . mg protein^{-1} . min^{-1} in the absence and presence of calmodulin, respectively. This increase of specific activity corresponds to a purification by a factor of ~100 as compared to the KCl-extracted microsomes and of 340 as compared to the crude microsomes [7]. The relative stimulation of the purified CaMgATPase by calmodulin is similar to the one in the KCl-extracted microsomes.

(5) The purified CaMgATPase is devoid of a MgATPase component (MgATPase is <1% of CaMgATPase). This implies that the MgATPase found in the KCl-extracted microsomes is due to other enzymes.

3.2. *The total activity and recovery of the ATPase*

Starting from 100 g frozen smooth muscle ~110 mg protein was obtained in the KCl-extracted microsomes and of this amount 34.1 ± 2.47 (6)% was solubilized by Triton X-100. Whereas the recovery of the MgATPase activity amounts to 33.9 ± 3.6 (6)%, a recovery of >100% is found for the CaMgATPase activity (i.e., 150 ± 9.7 (4)% and 130 ± 12.5 (5)% are found in the absence and presence of calmodulin, respectively). Such a high recovery might be due to the uncoupling effect of detergents on the Ca^{2+}-transport.

In the presence of the detergent, the intravesicular compartment no longer exists and therefore inhibitory high Ca^{2+}-gradients could not build up by the activity of the CaMgATPase.

The total amount of protein recovered by the final Ca^{2+}-free wash of the calmodulin gel is 0.80 ± 0.15 (5)% of the amount of solubilized protein put on the calmodulin gel. The total CaMgATPase activity recovered amounts to 9.3 ± 0.89 (4)% and 12.7 ± 1.80 (5)% in the absence and the presence of calmodulin, respectively. A considerable activity of calmodulin sensitive CaMgATPase in the Triton X-100 solubilizate does not bind to the calmodulin gel. This is indicated both by the presence of calmodulin-sensitive CaMgATPase in the breakthrough fractions, as well as by the low (~10%) recovery of the total CaMgATPase activity from the column. Readdition of the breakthrough fractions of the column to the calmodulin gel regenerated in the Ca form, resulted again in 10% binding of the CaMgATPase which could be released in Ca^{2+}-free medium. We are currently optimizing the conditions for CaMgATPase binding to the calmodulin gel.

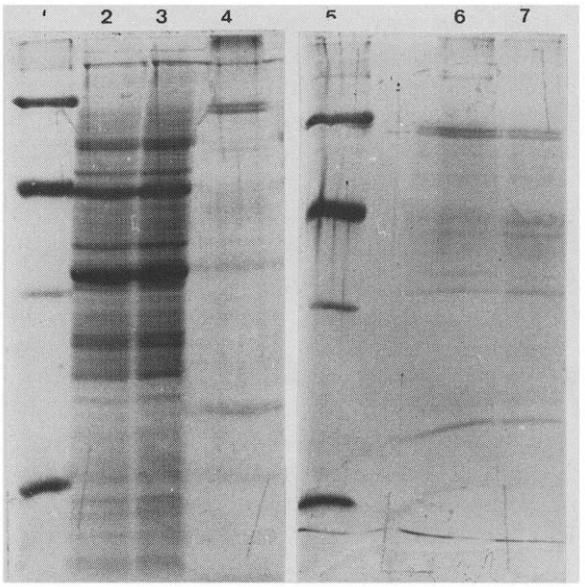

Fig.1. Two SDS gel electropherograms of the purified CaMgATPase (gel 1, lane 1–4; gel 2, lane 5–7); (1,5) M_r standards; (2) Triton X-100 solubilizate; (3) fraction not bound to calmodulin gel; (4,6,7) 3 different preparations of CaMgATPase. M_r standards 160 000, 68 000, 39 000 and 21 500. Proteins were stained with Coomassie brilliant blue.

3.3. *Gel electrophoresis*

Fig.1 shows a SDS-gel electrophoresis of the purified CaMgATPase. Several bands can be detected but the most prominent band has estimated M_r ~ 140 000. This is comparable to the value for CaMgATPase of human erythrocytes. We therefore postulate that this band corresponds to the CaMgATPase. This view is further supported by the observation that on gels run in an acid buffer [10], a Ca^{2+}-dependent phosphorylation is observed in a zone corresponding to M_r 140 000.

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References

[1] Wuytack, F., Landon, E., Fleischer, S. and Hardman, J. G. (1978) Biochim. Biophys. Acta 540, 253–269.
[2] Wuytack, F. and Casteels, R. (1980) Biochim. Biophys. Acta 595, 257–263.
[3] Wuytack, F., De Schutter, G. and Casteels, R. (1980) Biochem. J. 190, 827–831.
[4] Niggli, V., Penniston, J. T. and Carafoli, E. (1979) J. Biol. Chem. 254, 9955–9958.
[5] Gietzen, K., Tescka, M. and Wolf, H. U. (1980) Biochem. J. 189, 81–88.
[6] Wolf, H. U., Dieckvoss, G. and Lichtner, R. (1977) Acta Biol. Med. Germ. 36, 847–858.
[7] Wuytack, F., De Schutter, G. and Casteels, R. (1981) Biochem. J. in press.
[8] Peterson, G. L. (1977) Analyt. Biochem. 83, 346–356.
[9] Laemmli, U. K. (1970) Nature 227, 680–685.
[10] Stewart, P. S., MacLennan, D. H. and Shamoo, A. E. (1976) J. Biol. Chem. 251, 712–719.
[11] Sharma, R. K. and Wang, J. H. (1979) Adv. Cyclic Nucl. Res. 10, 187–198.