SINGLE STEP PURIFICATION OF THE CATALYTIC SUBUNIT(S) OF CYCLIC 3',5'-ADENOSINE MONOPHOSPHATE-DEPENDENT PROTEIN KINASE(S) FROM RAT MUSCLE

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SUMMARY

The catalytic subunit(s) of the cyclic 3',5'-adenosine monophosphate (cAMP) dependent protein kinase(s) from rat muscle has been purified from crude extract in a single step several thousand fold and with high yield. This was achieved by selective release and elution of the cationic catalytic part(s) from the anionic holoenzyme adsorbed to anion exchange cellulose by low amounts of cAMP. Evidence is presented for the existence of differently charged cationic catalytic subunits.

INTRODUCTION

The significance of protein phosphorylation is indicated by the many reports on the influence of cyclic nucleotides, which regulate the activity of the dependent protein kinases, on a series of biological control systems (1). In order to study protein phosphorylation, increasing use is being made of protein kinases to catalyze this type of reaction in vitro. However, procedures so far available to purifiy the appropriate enzymes are laborious and not efficient enough to allow its routine use as a tool. We describe here a simple method for the purification of the catalytic subunit(s) of the cyclic 3',5'-adenosine monophosphate (cAMP) dependent protein kinase(s) from rat muscle which is based on the following consideration. The literature values for the isoelectric points of cAMP dependent protein kinases from different mammalian tissues (2-6) suggest in general that the holoenzyme (CR) is a fairly acidic protein ($pI \leq 5.5$), whereas the subunits differ greatly with regard to their charge. At neutral pH the cAMP binding regulatory subunit (R) is anionic ($pI < 4$), the catalytic subunit (C), however, is cationic ($pI \geq 7.4$). Quite similar relations were found for the rat muscle enzyme (unpublished). From this consideration it follows that the intact holoenzyme bound to an anion exchanger should be split by treatment with cAMP according to the generally accepted equation

$$C \cdot R + cAMP \longrightarrow C + R \cdot cAMP.$$

In this way the catalytic subunit(s) should become selectively elutable by the cyclic nucleotide whereas the R·cAMP complex should stay on the column. In this paper we demonstrate that the enzyme can indeed be purified several thousand fold from crude muscle extract in a single step and in very high yield. The enzyme has the properties expected for the catalytic subunit of the cAMP dependent protein kinase.

MATERIALS AND METHODS

The following materials were obtained from the quoted sources: [γ-^{32}P] ATP (Sp. act.>10 Ci/mmole) and [^{3}H (G)] 3',5' cAMP (Sp.act. 37 Ci/mmole) from NEN, calf thymus histone type IIA and DEAE cellulose (0.85 mequiv/g medium mesh) from Sigma, protaminesulfate, the molecular weight standard proteins and the chemicals for gel-electrophoresis as well as gel-isoelectric focusing from Serva (Heidelberg, FRG). Ampholine was obtained from LKB, Sephadex G 200 from Pharmacia. The other chemicals used were of analytical grade. Skeletal muscle was obtained from female Sprague Dawley rats (250-400 g) and frozen until use. Protein kinase was assayed in 0.2 ml incubation volume modified according to Miyamoto et al. (7). The assay medium contained 50 mM sodium acetate buffer pH 6, 10 mM magnesium acetate, 40 μg histone, and 25 pmoles [γ-^{32}P] ATP. Some assays were done in the presence of 5×10^{-6} M cAMP. The reaction was stopped in an ice bath by the addition of 0.2 ml 2 mM ATP, 0.2 ml 0.63% bovine serum albumin solution and 4 ml 7.5% trichloroacetic acid (TCA). The sediment was resolubilized twice in 0.1 ml of ice cold 1 N NaOH and reprecipitated each time with 2 ml 5% TCA to remove remaining [γ-^{32}P] ATP (according to Greenaway, 8). The final precipitate was dissolved in 0.1 ml 1N NaOH and counted in 10 ml Aquasol (NEN). The data were not corrected for the overall recovery of radioactive protein (recovery was more than 80%). One unit of enzyme activity was defined as that amount of enzyme which transfers 1 pmole of ^{32}P from [γ-^{32}P] ATP to the recovered protein during 5 min at 30°C.
Electrophoresis in polyacrylamide gradient gels in the presence of sodium dodecylsulfate (SDS) was done essentially according to Laemmli (9) as described earlier (10). Isoelectric focusing in polyacrylamide gels and detection of focused enzyme in the gel using protaminesulfate as the phosphorus acceptor was carried out essentially according to Hirsch and Rosen (11) with minor modifications. Protein bound [^{3}H (G)] cAMP was measured mainly according to Kumon et al. (12). Protein measurement was done either by the method of Lowry et al. (13) or modified according to Schaffner and Weissmann (14) by measuring solubilized TCA precipitates which were stained with amido black at 630 nm. Bovine serum albumin was used as the standard.

RESULTS AND DISCUSSION

Enzyme purification was carried out at 4°. Frozen rat muscle was cut into small pieces and homogenized in 3 volumes 4 mM neutral EDTA containing 2 mM dithioerythritol (DTE) for 2 min in a cooled Waring Blender at highest speed. The homogenate was centrifuged at 25 000 x g_{max} for 30 min. The supernatant was filtered through glass wool, brought to pH 6.5 with 1 M dipotassium phosphate and dialysed against a total of 20 volumes 5 mM potassium phosphate buffer pH 6.5 containing 2 mM EDTA and 1 mM DTE for about 24 h with 2 changes of buffer. After dialysis the crude extract was clarified by filtration and immediately loaded on to a DEAE cellulose column containing 60 g of ionexchanger

per g of protein, which had been equilibrated with the above mentioned buffer. The column was washed with 0.15 M potassium phosphate buffer pH 6.5 containing 2 mM EDTA and 1 mM DTE until the eluate was free of detectable protein (usually about 5-6 bed volumes). The enzyme was eluted with 0.14 M buffer of otherwise the same composition as before but containing in addition 10^{-5} M cAMP. The fractions were collected in siliconized glass tubes. For routine use active fractions were pooled, and dialysed extensively against the 5 mM phosphate buffer mentioned above containing in addition 30-40 % glycerol. Storage was at 4^{o}.

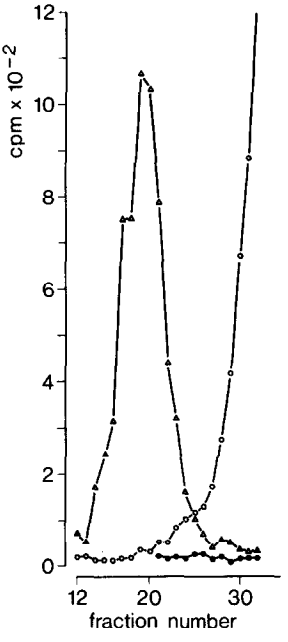


Figure 1: Protein kinase elution in relation to the appearance of cAMP in the eluate. Starting material: 19 g of rat muscle. Initial bed volume of the anion exchange column: 2.6x20.5 cm. Elution: 35 ml/h in 5.6 ml fractions. To the cAMP containing elution buffer 5 nCi/ml [^{3}H(G)] cAMP was added. Total tritium activity o—o; protein bound tritium •—•; protein kinase assay Δ—Δ.

A typical elution pattern is shown in Fig. 1. In this experiment some [^{3}H(G)] cAMP was added to elution buffer to visualize the appearance of the cyclic nucleotide in relation to the enzyme activity. Aliquots of each fraction were assayed for total tritium activity, protein bound tritium activity as well as for enzymatic activity. From the result it is evident that the enzyme peak precedes the appearance of the cyclic nucleotide, and that no protein bound

cyclic nucleotide can be detected. A subsequent elution of the column with 0.3 M phosphate buffer did not yield any protein kinase activity. According to Miyamoto et al. (7) as well as to our own experiments (15) a cAMP dependent enzyme would be expected to elute at that ionic strength. This indicates that the complete enzyme activity was released in the preceding step.

The enzyme released by cAMP is not stimulated by the cyclic nucleotide but rather inhibited approximately 10-15%. Similar observations have been made for the catalytic subunit by Erlichman at al. (16) and by Lemaire et al. (17).

The result of a typical enzyme purification is given in Table 1. Some difficulties were encountered in the calculation of enzyme yield, for the following reasons: (I) The crude extract contains inhibitors as seen from the yield calculation and as reported in the literature (18). This complicates the calculation of the true recovery of the enzyme. (II) Other substrate proteins in

Table 1: Summary of a single step purification of protein kinase. Starting material: 290 g rat muscle. Initial bed volume of the column: 5x43 cm. Elution: 100 ml/h. The value 9486 units in the crude extract represents the difference of the enzyme activities as measured in the presence and in the absence of cAMP (=$\Delta \pm$ cAMP)

	volume (ml)	protein (mg)	activity (units)	specific activity (units/mg)	purification (fold)	recovery (%)
crude extract	527	4216	9486	2.25	1	100
pooled eluate	128	0.558	28672	51383	22837	302

the crude extract (10) may influence the histone phosphorylation. (III) The crude extract contains in addition cAMP independent protein kinases, as found in the fraction washed through with the 0.15 M buffer. Since the purification procedure is based exclusively on the intact holoenzyme being bound to the ion exchanger, the fraction released by cAMP can only represent that amount of the catalytic subunit which was dissociated from the cAMP dependent enzyme. For this reason we chose as the basis for the calculations that part of the protein kinase activity in the crude extract which can be stimulated by cAMP. We therefore compared the difference in enzyme activity in the crude extract in the presence and in the absence of cAMP (=$\Delta \pm$ cAMP) with the activity of the

eluted enzyme as assayed without cAMP. From the yield estimation it follows that the degree of purification is not as high as it might appear from the data.

If the crude extract was frozen and thawed prior to the loading of the column, a procedure known to separate the subunits, the $\Delta\pm$ cAMP value was about 5 times lower and the amount of elutable enzyme accordingly smaller. From data published (7) it is not surprising that cGMP releases also protein kinase activity from the DEAE column.

Gel filtration of the enzyme on a Sephadex G 200 superfine column (1 x 40 cm) led to a rather broad peak of enzyme activity at a molecular weight of about 37 000. Electrophoresis of the enzyme in the presence of SDS showed, in addition to some minor impurities not visible on the photograph,a major band slightly above ovalbumin and a minor band at the level of ovalbumin (Fig. 2). It has also been reported that other basic proteins, like histones, do not migrate in SDS gels according to their molecular weights (19).

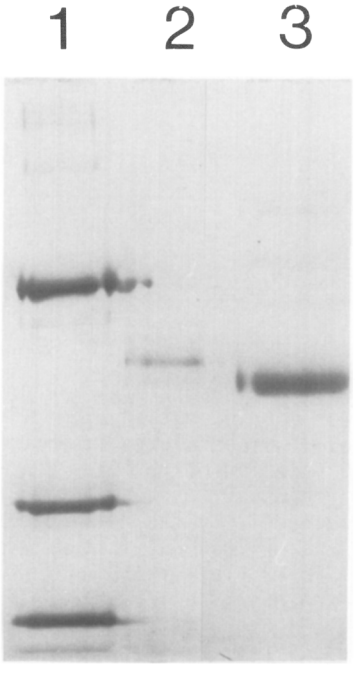
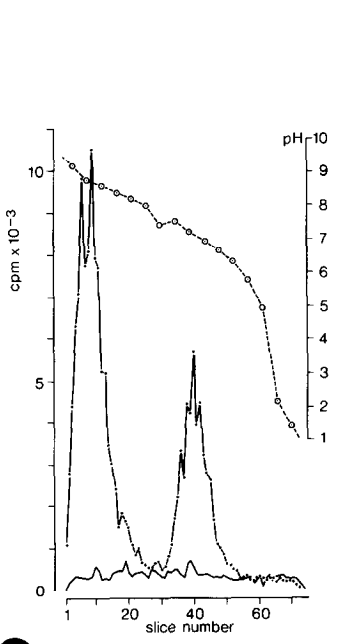
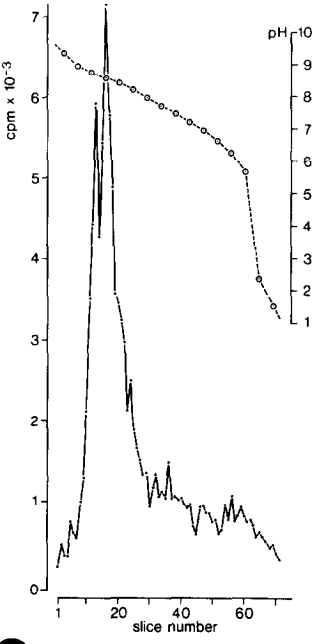


Figure 2: Gel electrophoresis of protein kinase in the presence of SDS. Gel electrophoresis in polyacrylamide gradients of 7.5 to 15% was performed as described in Ref. 9 and 10. The gels were stained with Coomassie blue. Slot 1 contains bovine serum albumin, chymotrypsinogen A and horse myoglobin (5µg of each), slot 2 protein kinase (5µg), slot 3 ovalbumin (5 µg).

Gel isoelectric focusing shows indeed that the enzymatic activity focuses also at basic pH in two major groups with main peaks at pH 8.6 and at approximately pH 7 (Fig. 3). If the complete purification is carried out at pH 7.5 instead of pH 6.5 the enzyme focusing at pH 7 is missing in the eluate as seen from Fig. 4. The yield is accordingly lower. The possibility that these patterns were caused by complex formations with nucleic acids is unlikely as demonstrated by preincubation of the protein kinase with 50 µg/ml of RNAase A or DNAase I.





Figures 3 and 4: Electrofocusing of protein kinase and enzyme assay in polyacrylamide gels. Focusing was carried out in 8 cm gels (5% acrylamide, 0,25% N,N'-methylenebisacrylamide, w/w) containing 2% Ampholine and 20% glycerol at 4° for 14-16 h applying 100 V permanently. After removing the gels and equilibrating the pH to 7 the enzyme assay was done at 30° for 30 min. After the extraction of unspecific radioactivity the gels were sliced, dried and counted. For pH determination one extra gel was cut into 0.5 cm slices, minced and suspended in distilled water. Fig. 3 (left): 150 units of protein kinase prepared at pH 6.5, assay in the presence of 9 µCi [γ-^{32}P] ATP, ·—·; control gel without enzyme,— . Fig. 4 (right): 65 units of protein kinase prepared at pH 7.5, assay in the presence of 22 µCi [γ-^{32}P] ATP.

The above data provide evidence that the catalytic subunit(s) of cyclic nucleotide dependent protein kinases can be indeed purified in a single step from crude muscle extract with high yields and several thousand fold purification

as theoretically expected. Most attempts, so far, to isolate the catalytic subunit(s) start from more or less purified holoenzyme which is separated into subunits either on affinity columns containing immobilized cAMP-analogues (20) or by the addition of cAMP prior to the separation on columns. Purification is then carried out either on affinity columns containing immobilized substrate protein (21, 4, 22) or on ion exchangers (23). Only one group has reported the elution of the catalytic subunit from prepurified holoenzyme bound to anion exchanger (16, 24) which was found on an empirical basis (25). At the present time, copurification of catalytic subunits from cGMP dependent protein kinases cannot be excluded but this is probably not a serious problem since the catalytic subunits of cAMP and cGMP dependent enzymes appear to have similar properties (4).

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REFERENCES

1. Advances in Cyclic Nucleotide Research (Greengard,P. and Robison, G.A. eds) Vol.I-VI, Raven Press, New York.
2. Chen, L.J. and Walsh, D.A. (1971) Biochemistry, 10, 3614-3621.
3. Gill, G.N. and Garren, L.D. (1971) Proc.Nat.Acad.Sci. USA, 68, 786-790.
4. Miyamoto, E., Petzold, G.L., Kuo, J.F. and Greengard, P. (1973) J.Biol.Chem. 248, 179-189.
5. Inoue, Y., Yamamura, H. and Nishizuka, Y. (1973) Biochem. Biophys.Res.Commun. 50, 228-236.
6. Nesterova, M.V., Sashchenko, L.P., Vasiliev, V.Y. and Severin, E.S. (1975) Biochim.Biophys.Acta 377, 271-281.
7. Miyamoto, E., Kuo, J.F. and Greengard, P. (1969) J.Biol.Chem. 244, 6395-6402.
8. Greenaway, P.J. (1972) Biochem.Biophys.Res.Commun. 47, 639-644.
9. Laemmli, U.K. (1970) Nature 227, 680-685.
10. Kinzel, V., Alonso, A. and Kübler, D. (1975) Eur.J.Biochem. 55, 361-368.
11. Hirsch, A. and Rosen, O.M. (1974) Anal.Biochem. 60, 389-394.
12. Kumon, A., Yamamura, H. and Nishizuka, Y. (1970) Biochem. Biophys.Res.Commun. 41, 1290-1297.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol.Chem. 193, 265-275.
14. Schaffner, W. and Weissmann, C. (1973) Anal.Biochem. 56, 502-514.
15. Kinzel, V. and Mueller, G.C. (1973) Biochim.Biophys.Acta, 322, 337-351.

16. Erlichman, J., Hirsch, A.H. and Rosen, O.M. (1971) Proc.Nat. Acad.Sci. USA, 68, 731-735.
17. Lemaire, S., Labrie, F. and Gauthier, M. (1974) Can.J. Biochem. 52, 137-141.
18. Walsh, D.A., Ashby, C.D., Gonzalez, C., Calkins, D., Fischer, E.H. and Krebs, E.G. (1971) J.Biol.chem. 246, 1977-1985.
19. Panyim, S. and Chalkley, R. (1971) J.Biol.Chem. 246, 7557-7560.
20. Wilchek, M., Salomon, Y., Lowe, M. and Selinger, Z. (1971) Biochem.Biophys.Res.Commun. 45, 1177-1184.
21. Reimann, E.M., Brostrom, C.O., Corbin, J.D., King, C.A. and Krebs, E.G. (1971) Biochem.Biophys.Res.Commun. 42, 187-194.
22. Corbin, J.D., Brostrom, C.O., King, C.A. and Krebs, E.G. (1972) J.Biol.Chem. 247, 7790-7798.
23. Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1974) in: Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. eds.) Vol.38 pp. 299-308, Academic Press, New York.
24. Rubin, C.S., Erlichman, J. and Rosen, O.M. (1974) in: Methods in Enzymology (Colowick, S.P. and Kaplan, N.O. eds.) Vol.38 pp. 308-315, Academic Press, New York.
25. Rosen. O.M., Erlichman, J. and Rubin, C.S. (1975) in: Advances in Cyclic Nucleotide Research (Drummond, G.I. Greengard, P. and Robison, G.A., eds.) Vol.5 pp. 253-263, Raven Press, New-York.