Vol. 84, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1978 Pages 777-785

DISSOCIATION AND REASSOCIATION OF A PIG HEART PHOSPHOPROTEIN PHOSPHATASE*

Michinori Imazu, Teiji Imaoka, Hirofumi Usui and Masao Takeda

Department of Biochemistry
Hiroshima University School of Medicine, Hiroshima 734, Japan

Received August 31, 1978

SUMMARY: A pig heart phosphoprotein phosphatase with a molecular weight of 224,000 was dissociated in the presence of 40 % ethanol into an active component (C) of molecular weight 31,000 and components (R) of higher molecular weight. After removal of the ethanol, C and R reassociated and formed an enzyme of molecular weight 188,000. C alone could not form the enzyme. The newly formed enzyme had substrate specificity and response to Mg acetate similar to those of the original large form of the enzyme and was clearly distinguishable from C. The ability of R to associate with C was supressed by treatment of R with trypsin or heat (60°C, 2 min), but not with RNase or DNase.

A phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16), which dephosphorylates phosphorylase a (EC 2.4.1.1), glycogen synthetase D (EC 2.4.1.11), phosphorylase b kinase (EC 2.7.1.38) and other proteins phosphorylated by cyclic AMP[1/]-dependent protein kinase (EC 2.7.1.37), exists in multiple molecular forms in extracts of various mammalian tissues (1-8). Brandt et al. (9) have demonstrated that the multiple forms of the enzyme in various rat tissue extracts may be converted by 80 % ethanol treatment to a single small component of molecular

* This investigation has been supported in part by research grants from Naito Science Foundation (1976), and Scientific Research Fund of the Ministry of Education of Japan (1976-1978).

1/ The abbreviation used are: Cyclic AMP, adenosine 3', 5'-monophosphate; ^{32}P-H2B histone, H2B histone phosphorylated with cyclic AMP-dependent protein kinase; ^{32}P-H1 histone, H1 histone phosphorylated with cyclic AMP-dependent protein kinase; DTT, dithiothreitol.

0006-291X/78/0843-0777$01,00/0

Copyright © 1978 by Academic Press, Inc.
All rights of reproduction in any form reserved.

weight 30,000-35,000. Although such conversion of cardiac phosphoprotein phosphatases has also been reported (10, 11), the reconstitution of high molecular weight forms from the small form has not been successful.

In this paper, we report the dissociation, by 40 % ethanol treatment, of a pig heart phosphoprotein phosphatase (original large form, Mr = 224,000) into an active component (small form, C, Mr = 31,000), and other components (R) of higher molecular weight. The reassociation of C and R produced an enzyme form of molecular weight 188,000 (reconstituted large form). The reconstitution of the high molecular weight form from the small form was accompanied by pronounced changes in substrate specificity as well as other properties of the enzyme. These results strongly suggested that the high molecular weight form was an associated form of the small form with other components and was not an oligomeric form of the small form alone.

MATERIALS AND METHODS

Calf thymus whole histone, H2B histone and H1 histone were prepared as described previously (12). Cyclic AMP-dependent protein kinase was purified from pig heart by the method of Rubin et al. (13). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (14). Other materials and chemicals were obtained from commercial sources.

^{32}P-H2B and H1 histones were prepared with [γ-^{32}P]ATP and pig heart cyclic AMP-dependent protein kinase as described previously (12). ^{32}P-H2B histone, used in these studies, contained 65 % of total alkali-labile phosphate (63 nmol/mg of H2B histone, 10^4 cpm/nmol) at Ser-36 and 32 % at Ser-32. ^{32}P-H1 histone contained 17 nmol alkali-labile phosphate (10^5 cpm/nmol) at Ser-38 per mg of H1 histone. All substrate concentrations represent the concentration of alkali-labile phosphate moiety of the substrate protein.

Phosphoprotein phosphatase was routinely assayed by measuring [^{32}P]orthophosphate which was released from ^{32}P-H2B histone. The reaction mixture (0.1 ml), containing 50 μM ^{32}P-H2B histone, 50 mM Tris-HCl, pH 7.2, 50 mM Mg acetate and 0.5 mM DTT, was incubated for 10 min at 30°C and [^{32}P]orthophosphate release was determined as described previously (11). When ^{32}P-H1 histone (25 μM) was used as a substrate, Mg acetate was replaced by 200 mM NaCl. One unit of the enzyme was defined as the amount of

enzyme which catalyzed the release of 1 nmol of [^{32}P]orthophosphate per min. Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

Phosphoprotein phosphatase was purified from pig heart as described previously (11). The enzyme preparation could be stored on ice for at least one week before use in experiments.

Molecular weights and frictional ratios (f/fo) of proteins were determined by the methods of Siegel and Monty (16) from sedimentation coefficients ($s_{20,w}$) and Stokes radii. Sedimentation coefficients were determined by sucrose gradient analysis by the method of Martin and Ames (17) with beef liver catalase (11.3 S), yeast alcohol dehydrogenase (7.4 S) and horse heart cytochrome c (1.9 S) as standards. The Stokes radii were estimated by gel filtration with either Sephadex G-150 or Sephadex G-200 columns with catalase (5.2 nm), human-γ-globulin (5.55 nm), bovine serum albumin (3.5 nm) and ovalbumin (2.73 nm) as standards under conditions specified earlier (11).

RESULTS AND DISCUSSION

A previous paper (11) has described the conversion of a high molecular weight form (large form, apparent Mr ≥ 250,000) of the phosphoprotein phosphatase from pig heart to a smaller form (small form, apparent Mr = 30,000) by treatment with 80 % ethanol. During this procedure, however, about half of the enzyme protein was insolubilized. To prevent the irreversible denaturation of the enzyme, a minimum ethanol concentration (40 %) was employed to dissociate the enzyme (unless otherwise stated, all procedures were carried out below 4°C). To one ml of the large form of the enzyme (345 units, 15 mg protein), 0.73 ml of 95 % ethanol was gradually added with gentle stirring within 6 min. The mixture was immediately centrifuged for 5 min at 30,000 x g. The precipitated protein (about 4 % of the total protein) was discarded. The supernatant solution was then diluted with the same volume of 50 mM Tris-HCl, pH 7.4 containing 1.0 mM DTT, and the mixture was directly applied to a Sephadex G-150 column equilibrated with the same buffer under the conditions given in Fig. 1. More than 80 % of ^{32}P-H2B histone phosphatase activity was recovered in peak 3 (Fig. 1B). The rest of the activity was eluted as minor

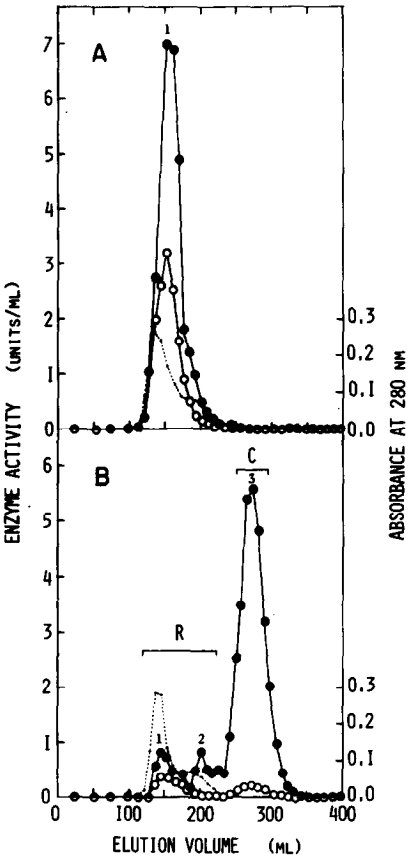

Fig. 1. Gel filtration of phosphoprotein phosphatase on a Sephadex G-150 column. A, before ethanol treatment; B, after ethanol treatment. The purified enzyme (345 units, 15 mg protein) before or after ethanol treatment (see text) was applied to a Sephadex G-150 column (2.5 x 80 cm) equilibrated with 50 mM Tris-HCl, pH 7.4 containing 1 mM DTT. Elution was performed upward with the same buffer and fractions of 3.8 ml each were collected at a flow rate of 18 ml/hr. Enzyme activity was determined as described under MATERIALS AND METHODS. Solid lines with ● and o indicate enzyme activity assayed with ^{32}P-H2B histone and ^{32}P-H1 histone, respectively. The dotted line indicates absorbance at 280 nm.

peaks (peaks 1 and 2) in the fractions of the higher molecular weight range. Fig. 1A represents an elution profile of the untreated enzyme. The molecular weights of the untreated enzyme and peak 3 in Fig. 1B were estimated to be 224,000 and 31,000, respectively, from sedimentation coefficients and Stokes radii (Table I). The calculated frictional ratios (f/fo) indicated

Table I. Properties of various molecular forms of a pig heart phosphoprotein phosphatase

Enzyme	$s_{20,w}$ [a] (S)	Stokes radius [a] (nm)	Molecular weight [a]	Activity ratio H2B/H1 [b]	Activity ratio $+Mg^{2+}/-Mg^{2+}$ [c]
Original large form	7.7 ±0.1	6.8 ±0.3	224,000 ±8,000	2.2	5.2
Small form	3.5 ±0.1	2.1 ±0.1	31,000 ±2,000	34.0	1.6
Reconstituted large form	7.4 ±0.1	6.0 ±0.2	188,000 ±7,000	2.4	2.6

a/ The mean ± S.D. of data of experiments with more than three separate analyses are given.
b/ ^{32}P-H2B histone and ^{32}P-H1 histone phosphatase activities were measured as described under MATERIALS AND METHODS.
c/ ^{32}P-H2B histone phosphatase activities with and without 50 mM Mg acetate were measured as described under MATERIALS AND METHODS.

that the original large form was asymmetric, possessing f/fo of 1.70, whereas the small form was essentially an unhydrated sphere, with f/fo equal to 1.01.

The conversion of the large form of the enzyme to the small form by 80 % ethanol treatment was accompanied by an alteration of substrate specificity and response to Mg acetate (18). Therefore, the ratio of enzyme activities for ^{32}P-H2B histone and ^{32}P-H1 histone (activity ratio H2B/H1), and that with and without 50 mM Mg acetate (activity ratio $+Mg^{2+}/-Mg^{2+}$) were employed to monitor the two forms of the enzyme. The activity ratios H2B/H1 and $+Mg^{2+}/-Mg^{2+}$ of the original large form (peak 1

in Fig. 1A) were 2.2 and 5.2, respectively, whereas, those of peak 3 in Fig. 1B were 34 and 1.6, respectively (Table I). These results indicated that more than 80 % of the original large form of the enzyme was converted to the small form upon treatment with 40 % ethanol.

In order to test whether the dissociated components of the large form of the enzyme could reassociate after removal of the ethanol, the fractions designated as R (100 ml) and C (47 ml) in Fig. 1B were separately pooled and concentrated to 2 ml by an Amicon ultrafiltration cell equipped with a YM-10 filter membrane. The mixture of fractions R and C (2 ml each), and each fraction (2 ml) were left standing for 30 min at 4°C and chromatographed on three Sephadex G-150 columns, separately (Fig. 2). Fig. 2A shows an elution profile of a mixture of R and C. A significant amount of a large form of the enzyme (peak 1) was eluted in addition to the small form of the enzyme (peak 2). The reconstituted large form had a molecular weight of 188,000 and a f/fo of 1.58. It showed activity ratios H2B/H1 and $+Mg^{2+}/-Mg^{2+}$ of 2.4 and 2.6, respectively, which were similar to those of the original large form (Table I). As shown in Fig. 2B, 95 % of the applied histone phosphatase activity of C was eluted as the small form. This indicated that the small form alone could not reassociate with each other to an oligomeric form of higher molecular weight. Although the residual histone phosphatase activity associated with R was eluted in similar fractions (peak 2 in Fig. 2C) as the reconstituted large form, it was only 35 % of the activity of the reconstituted large form which was eluted as peak 1 in Fig. 2A. The association of R with C seems to be specific, since R could not be substituted by other proteins such as human-γ-globulin.

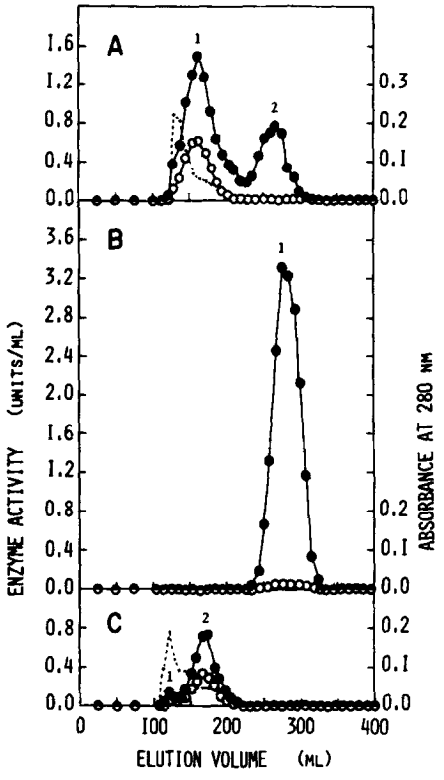

Fig. 2. Gel filtration of R and C on a Sephadex G-150 column. A, R and C; B, C alone; C, R alone. R (38 units, 9.1 mg protein) and/or C (150 units, 0.1 mg protein) were applied to a Sephadex G-150 column (2.5 x 80 cm). Gel filtration and determination of enzyme activity were performed as described in the legend to Fig. 1. Solid lines with • and o indicate enzyme activity assayed with ^{32}P-H2B histone and ^{32}P-H1 histone, respectively. The dotted line indicates absorbance at 280 nm.

Table II shows that R and C synergistically increased their ^{32}P-H1 histone phosphatase activities. Treatment of R with RNase or DNase did not affect the synergistic effect. After incubation of R with trypsin, the synergistic effect was supressed. This experiment indicated that the stimulatory activity of R was associated with a protein and not with a nucleic acid. The stimulatory activity of R was heat labile. Exposure of R to 60°C for 2 min markedly reduced its activity.

Table II. Nature of R from a pig heart phosphoprotein phosphatase

Treatment of R	^{32}P-H1 histone phosphatase activity[a] (unit x 10^3)			Increase in activity[d]
	(R+C)	(R)[b]	(C)[c]	(%)
None	464	224	33	45
RNase	440	227	34	41
DNase	451	225	40	41
Trypsin	181	84	64	18
Heat (60°C, 2 min)	88	39	33	18

a/ The reaction mixture (100 µl) contained 50 mM Tris-HCl, pH 7.2, 1 mM Mg acetate, 1 mM DTT, 0.4 mg protein of R, and when applicable, 6 µg of RNase (Sigma), DNase (Sigma), or trypsin (Worthington). At the end of 30 min at 30°C, 0.6 mg of trypsin inhibitor (Sigma) was added to a tube which contained trypsin. For heat treatment, the reaction mixture was heated at 60°C for 2 min. C (2.23 units with ^{32}P-H2B histone, 4 µg protein) in 100 µl of 50 mM Tris-HCl, pH 7.2 containing 1 mM DTT was added and mixtures of R and C were standing for 30 min on ice. Five µl of the mixture were taken and the ^{32}P-H1 histone phosphatase activity was assayed as described under MATERIALS AND METHODS.
b/ Tubes containing no C.
c/ Tubes containing no R.
d/ Calculated from the formula: [(R+C)-(R)-(C)] x 100/(R+C)

Since association of R with C changed substrate specificity and responce to Mg acetate of C, R might play a role in regulating C activity as regulatory subunits.

The reason why the original enzyme form of molecular weight 224,000 has not been reconstituted, is unknown. Other components which were inactivated or lost during the 40 % ethanol treatment may be necessary for the conversion of the newly produced form of molecular weight 188,000 to the original form of molecular weight 224,000.

Acknowledgements: The authors are grateful to Professor Yasutomi Nishizuka (Kobe University) for his criticism and suggestion during the preparation of this manuscript. Thanks are also due to Miss Mieko Kawamura and Miss Noriko Nishimura for their secretarial and technical assistance.

REFERENCES

1. Meisler, M.H., and Langan, T.A. (1969) J. Biol. Chem. 244, 4961-4968.
2. Abe, N., and Tsuiki, S. (1974) Biochim. Biophys. Acta 350, 383-391.
3. Kato, K., and Sato, S. (1974) Biochim. Biophys. Acta 358, 299-307.
4. Thomas, J.A., Young, T., and Mellgren, R. (1974) Fed. Proc. 33, 1315.
5. Li, H.-C. (1975) FEBS Lett. 55, 134-137.
6. Ray, K.P., and England, P.J. (1976) Biochem. J. 157, 369-380.
7. Antoniw, J.F., Nimmo, H.G., Yeaman, S.J., and Cohen, P. (1977) Biochem. J. 162, 423-433.
8. Paletta, E., and Sands, H. (1978) Biochim. Biophys. Acta 523, 121-132.
9. Brandt, H., Killilea, S.D., and Lee, E.Y.C. (1974) Biochem. Biophys. Res. Commun. 61, 548-554.
10. Hsiao, K.-J., Chan, W.W.S., and Li, H.-C. (1977) Biochim. Biophys. Acta 483, 337-347.
11. Imaoka, T., Imazu, M., Ishida, N., and Takeda, M. (1978) Biochim. Biophys. Acta 523, 109-120.
12. Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K., and Iwai, K. (1976) J. Biol. Chem. 251, 6287-6293.
13. Rubin, C.S., Erlichman, J., and Rosen, O.M. (1972) J. Biol. Chem. 247, 36-44.
14. Glynn, I.M., and Chappell, J.B. (1964) Biochem. J. 90, 147-149.
15. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Siegel, L.M., and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346-362.
17. Martin, R.G., and Ames, B.N. (1961) J. Biol. Chem. 236, 1372-1379.
18. Usui, H., Imazu, M., Imaoka, T., and Takeda, M. (1978) Biochim. Biophys. Acta, in press.