HETEROGENEITY OF THE CATALYTIC ACTIVITY ARISING FROM THYROIDAL cAMP-DEPENDENT PROTEIN KINASE TYPE II

D. TORU-DELBAUFFE, R. OHAYON and M. PAVLOVIC-HOURNAC

Unité de Recherche sur la Glande Thyroide et la Régulation Hormonale, INSERM, Unité 96, CNRS, ER 233, 78, rue du Général Leclerc, 94270 Bicetre, France

Received 18 December 1981

1. Introduction

It is now generally agreed that the 2 types of cyclic AMP-dependent protein kinases (PK I and PK II) are distinct mostly with respect to their regulatory subunits (R), which have different physicochemical (e.g., charge, M_r-value) and immunological properties [1–6]. Their peptide maps are entirely different [7]. The catalytic subunits (C) are, however, considered as almost identical, since they have similar, if not identical, M_r-values, enzymatic characteristics, substrate specificities and immunological determinants [1,6,8–12]. In spite of this great similarity, isoelectric points of catalytic subunits arising from both PK are heterogenous [9,13–15]. A very small difference was also noted in their amino acid composition and primary structure [7]. Some data suggest that the 2 PK from several organs could have different stability upon hormonal and other treatments [16–19], but it is not known whether this is due to the properties of regulatory or catalytic subunits.

Here, we have studied the stability of the catalytic activity after dissociation of the 2 PK with cAMP. Data are presented showing the heterogeneity concerning the stability of the catalytic subunit population arising from PK II after dissociation of holoenzyme by cAMP.

2. Materials and methods

2.1. *Preparation of protein kinases*

Glands from 5–7 male Sprague Dawley rats were homogenized in an all glass homogenizer (Potter Dual 22) in 15 vol. (w/v) of either TKM buffer (50 mM Tris–HCl (pH 7.4) containing 5 mM $MgCl_2$, 25 mM KCl, 6 mM β-mercaptoethanol and 0.25 M sucrose) or TEM buffer (5 mM Tris–HCl (pH 7.4), 3 mM EDTA and 6 mM β-mercaptoethanol). Homogenates were centrifuged for 2 h at 50 000 rev./min and supernatants representing the cytosolic fraction, were removed and analyzed either directly or after fractionation by sucrose gradient ultracentrifugation.

2.2. *Fractionation of protein kinases by sucrose gradient ultracentrifugation*

The samples of cytosols (1–4 mg protein in 1 ml) were layered on the top of a 12 ml linear sucrose gradient (11–22% sucrose) and centrifuged in a Spinco rotor SW 40Ti at 38 000 rev./min for 41 h at 0°C; gradients were fractionated automatically into 0.3 ml fractions by a density gradient fractionator. Under these conditions thyroidal cAMP-dependent protein kinases were recovered in 2 peaks: type I kinase corresponds to peak 1 which sediments at 4.9 S; type II kinase corresponds to peak 2, which sediments at 6.2 S [18,20]. For the estimation of sedimentation coefficients, myoglobin ($s_{20,w}$ = 2) and glucose-oxidase ($s_{20,w}$ = 7.93) were used as internal markers. The yield of protein kinase activity after sucrose gradient centrifugation was 80–120%.

2.3. *Estimation of protein kinase activity*

Aliquots of cytosolic enzymes (10–30 μl) or of sucrose gradient fractions (50–80 μl) were incubated in 200 μl (final vol.) of medium containing: 50 mM β-glycerophosphate; 0.3 mM EGTA; 2 mM theophylline and 10 mM magnesium acetate in 1 mM potassium phosphate buffer (pH 7.4); [γ-^{32}P]ATP was added at

Abbreviations: TSH, thyrotropin; DBC, dibutyryl cyclic AMP; EDTA, ethylene diaminotetraacetic acid; EGTA, ethylene-bis-(β-amino-ethyl-ether)-*N,N'*-tetraacetic acid

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

0.1 mM (spec. act. 90–100 cpm/pmol). Histones were at 4 mg/ml, when added, cyclic AMP, at 5 μM, and DBC at 0.25 mM. In all assays with cAMP, methylisobutylxanthine was added at 0.1 mM. Incubations were done at 30°C for 5 or 10 min. At the end of the incubation 150 μl of the incubation mixture was spotted onto 2 cm discs of Whatmann 3 MM filter paper, which were thereafter processed as in [18].

Since we have found that rat thyroid cytosol contains a substantial amount of cAMP-independent histone kinase [20], we estimated both cAMP-dependent and cAMP-independent activities in parallel throughout this study. Cyclic AMP-independent histone kinase activity was evaluated in the presence of the heat-stable inhibitor of cAMP-dependent enzymes [21] which was prepared in the laboratory from hog or sheep brains and purified up to the Sephadex G-75 filtration step [22].

2.4. *Expression of results*

The cAMP-dependent activity described here refers to the net activity which was calculated as follows: activity measured in the presence of cAMP from which the cAMP-independent activity was subtracted.

All results were expressed either as cpm or pmol ^{32}P incorporated into histone . mg gland (wet wt)$^{-1}$. min^{-1} or 5 min^{-1} incubation, or as activity ratio (–cAMP/+cAMP activity).

3. Results

The stability of the catalytic activity was studied in enzyme samples at different time intervals after dissociation.

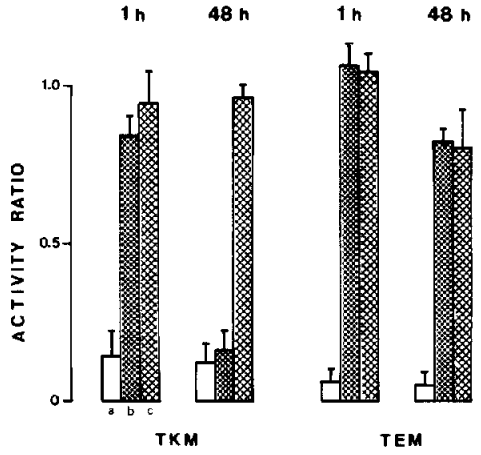

Fig.1. Maintenance of the dissociation of cytosolic cAMP-dependent protein kinases. Cytosols prepared either in TKM or TEM buffer were incubated with: (a) none; (b) cAMP (5×10^{-6} M) + 1-methyl-3-isobutylxanthine (10^{-4} M); (c) DBC (2.5×10^{-4} M) at 4°C. Protein kinase activities were estimated after 1 h and 48 h incubation. Results are expressed as activity ratios. Mean ± SEM of 3–8 separate expt; for each experiment cytosols from 5–10 glands were pooled.

3.1. *Maintenance of dissociation*

The maintenance of dissociation can be followed by determination of the activity ratios upon treatment of enzymes by cAMP. By using 2 different buffers (TEM and TKM) the dissociated state can be maintained indefinitely (fig.1). In TEM buffer the dissociation was well preserved for up to 48 h, while in TKM buffer it was less stable and led to the reassociation of regulatory and catalytic subunits into the inactive complex (R_2C_2), probably because of the lower stability of cAMP in this buffer. However, if cAMP was

Table 1
Protein kinase activities and activity ratios 48 h after dissociation of cytosols by cAMP and DBC

Dissociating agent	TKM buffer		TEM buffer	
	PK activity[a]	Activity ratio[b]	PK activity	Activity ratio
None	100 ± 14[c]	0.13 ± 0.05	100 ± 28	0.06 ± 0.03
cAMP	77 ± 12	0.18 ± 0.04	45 ± 11[e]	0.82 ± 0.03
DBC	52 ± 5[d]	0.94 ± 0.03	45 ± 4[e]	0.79 ± 0.14

[a] Protein kinase activity (pmol ^{32}P . mg^{-1} . min^{-1}) was expressed in % of control
[b] Activity ratio = –cAMP/+cAMP activity
[c] Mean ± SEM of 3–8 separate expt: for each experiment cytosol from 5–10 glands were pooled
[d] $P < 0.02$; [e] $P < 0.001$

replaced by DBC, the dissociation can be maintained for ≥48 h in both buffers.

3.2. *Loss of activity after dissociation*

Aliquots of cytosol prepared either in TKM or TEM buffer were activated with cAMP or DBC and their total activity was measured 48 h after dissociation. As can be seen from table 1, the total activity was significantly decreased (~50%) in all samples in which the dissociation was maintained (high activity ratios) throughout the period of observation. In samples in which the dissociation was not maintained (low activity ratios; samples incubated with cAMP in TKM buffer), the total activity was almost unchanged with respect to control, non-treated assays. It seems therefore that there is a parallelism between the maintenance of the high activity ratios and the loss of the enzyme activity.

To see if the decrease of kinase activities, observed after dissociation of enzymes is due to the loss of activity of both, or only of 1 of the 2 types of protein kinases, the following experiments were designed:

3.2.1. Sucrose gradient ultracentrifugation pattern of reassociated enzymes

Cytosol, prepared in TKM was activated by cAMP and analyzed by sucrose gradient ultracentrifugation. During centrifugation all protein kinase activity was reassociated, i.e., found in the form of holoenzymes. As can be seen in fig.2, the pattern of cytosol, dissociated before centrifugation, was different from the pattern of control, non-dissociated aliquot. While the activity of the PK I was not changed in cytosol submitted to the action of cAMP (104 ± 22% with respect to control), a visible decrease of the second peak was noted (46 ± 10% with respect to control), suggesting that it may have a lower stability upon dissociation.

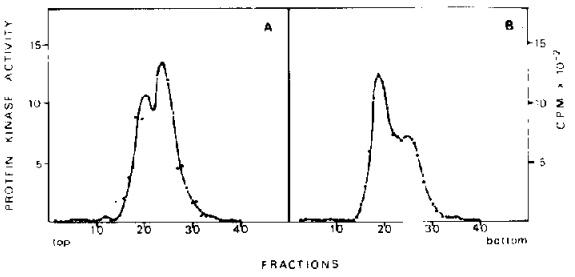

Fig.2. Sucrose gradient ultracentrifugation patterns of cytosols incubated without (A) and with cAMP (B). Cytosols were incubated with or without cAMP for 1 h at 4°C prior to centrifugation. In the assay incubated with cAMP protein kinases were completely activated (activity ratio = 0.96) before centrifugation. After centrifugation they were, however, found in the reassociated form.

3.2.2. Dissociation of the individual peaks of PK I and PK II

The stability of the catalytic activity arising from PK I and PK II was evaluated upon their activation by cAMP or DBC. The analysis of samples of individual peaks, showed that after dissociation, PK I lost only ~10% of activity in 48 h, while the activity of PK II decreased >30% (table 2).

In the next type of experiment the stability of the catalytic activities arising from the 2 types of kinases was evaluated during activation of enzymes with increasing concentrations of cAMP. Aliquots of type I and II were incubated in TEM buffer for 1 h at 4°C with cAMP. Total activity and activity ratios were measured after further incubation of aliquots for 5 min at 30°C in the presence of histones.

The activity of the PK I remains stable up to 50% activation (activity ratio = 0.5); thereafter a slight loss of activity was observed, which was ≤10% when total activation was reached (activity ratio = 1). On the contrary, the activity of PK II starts to decrease very early, as soon as the enzyme undergoes the action of cAMP (activity ratio = 0.2). After total activation (activity ratio = 1) the PK II lost ~30% of its activity (fig.3). These values are very similar to those obtained 48 h after dissociation (table 2). It seems therefore, that the activation of the 2 forms of PK induces a loss

Table 2
Loss of protein kinase activity measured 48 h after dissociation of individual peaks of PK I and PK II

Sample	Activity (% of control)	
	PK I	PK II
Control (non-dissociated)	100 ± 15 (5)[a]	100 ± 14 (6)
Dissociated[b] with cAMP or DBC	88 ± 5 (5)	67 ± 3 (6)

[a] Mean ± SEM of 5–10 separate expt: for each experiment glands from 5–10 rats were pooled

[b] Aliquots of PK I and PK II in TEM buffer were dissociated either by cAMP or DBC. Dissociation was maintained for 48 h (activity ratios = 0.93–0.95) and protein kinase activity was then evaluated

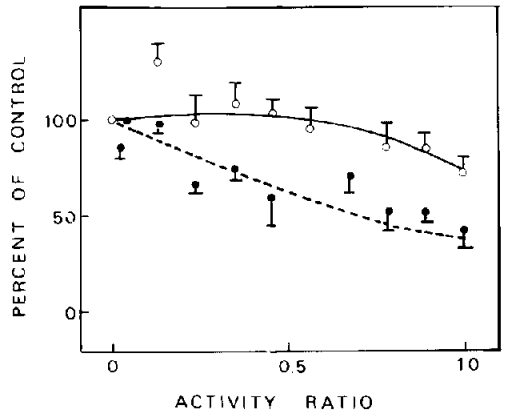

Fig.3. Differential loss of catalytic activity upon dissociation of PK I and PK II with increasing doses of cAMP. Results are represented with respect to the degree of dissociation, and expressed in % of control non-dissociated aliquots. The same number of units were used for each one of enzymes. The dose of cAMP necessary for the half-maximal activation (activity ratio = 0.5) was the same (3×10^{-8} M) for both PK I and PK II. The differences between the PK I and PK II became highly significant ($P < 0.02$) as soon as the activity ratios reached the value of 0.3.

of activity, which is obviously more important for the catalytic subunits arising from PK II than from PK I. According to our results, the loss is apparently achieved almost in totality after 1 h incubation, since it was not further increased between 1 h (fig.3) and 48 h (table 2) of dissociation.

4. Discussion

Our previous data had suggested that in thyroid glands PK II has apparently a greater instability than PK I; however from these data it was not possible to know if the more rapid decrease of PK II after some treatments was due to the selective degradation of the regulatory or of the catalytic subunits [18]. These results show that, upon dissociation, a part of the catalytic activity arising from PK II is selectively lost. This loss which represents ~30% of the total activity, was observed as soon as the enzyme was exposed to low doses of cyclic nucleotide (activity ratio = 0.2–0.3) and its maximum was reached already during the first 1 h following dissociation. It was not significantly enhanced when dissociation was maintained for 48 h or more.

In spite of the belief that the catalytic subunits from the 2 PK isoenzymes are not distinct, our results suggest that the catalytic activity from PK II may be heterogenous and contain a population of subunits which is inactivated rapidly upon dissociation. In the purified preparations this population was not found probably because of its high lability, which led to its selective loss from the first steps of purification. Because of its high instability, it is difficult to know whether this population of catalytic subunits has the same enzymatic and immunological properties as those described for the pure preparation, but even if so, its rapid turnover could be ascribed to some other minor differences either in its primary structure or configuration which are difficult to detect. The observed heterogeneity of the isoelectric points, is, for example not well understood [6].

The mechanism of this selective inactivation is also unknown. Whether it is due to the action of the heat stable regulator [21,23] or some very specific protease [24] is hard to say, and it will be very difficult to find. It could be, however, interesting to point out that this factor(s) copurifies with PK II and may even be intrinsic to the enzyme.

However, independent of the mechanism of its inactivation, this population of catalytic subunits may have a very important role in the cell in the fine regulation of some phosphorylation. Its effect being very brief, and taking place at very low concentrations of cAMP, may signify that this kinase is involved in the maintenance and regulation of the basal activity of some cellular processes. Indeed, in thyroid glands the 'tonic effect' of very low doses of TSH was reported [25]. It was found that the regulation of resting thyroid function by TSH apparently involves parallel changes in adenylate cyclase and protein kinase activities. Therefore these enzymes may well be the primary loci for the observed tonic effect of TSH [25]. In addition, the necessity of the permanent presence of very low doses of TSH in thyroid cell culture system, for a good response to higher, stimulatory doses of TSH, has been reported [26].

Acknowledgements

The authors wish to thank Mrs C. Sais for secretarial assistance. This work was partly supported by research contract 776 of UER, Kremlin, Bicetre.

References

[1] Erlichman, J., Rubin, C. S. and Rosen, O. M. (1973) J. Biol. Chem. 248, 7607–7609.

[2] Corbin, J. D., Keely, S. L. and Park, C. R. (1975) J. Biol. Chem. 250, 218–225.

[3] Rosen, O. M. and Erlichman, J. (1975) J. Biol. Chem. 250, 7788–7794.

[4] Hofmann, F., Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1975) J. Biol. Chem. 250, 7795–7801.

[5] Fleischer, N., Rosen, O. M. and Reichlin, M. (1976) Proc. Natl. Acad. Sci. USA 73, 54–58.

[6] Nimmo, H. G. and Cohen, P. (1977) Adv. Cyclic Nucl. Res. 8, 145–266.

[7] Zoller, M. J., Kerlavage, A. R. and Taylor, S. S. (1979) J. Biol. Chem. 254, 2408–2412.

[8] Rubin, C. S., Erlichman, J. and Rosen, O. M. (1972) J. Biol. Chem. 247, 36–44.

[9] Yamamura, H., Nishiyama, K., Shimomura, R. and Nishizuka, Y. (1973) Biochemistry 12, 856–862.

[10] Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1975) Adv. Cyclic Nucl. Res. 5, 241–251.

[11] Taylor, S. S. and Stafford, P. H. (1978) J. Biol. Chem. 253, 2284–2287.

[12] Schwoch, G., Hamann, A. and Hilz, H. (1980) Biochem. J. 192, 223–230.

[13] Chen, L. J. and Walsch, D. A. (1971) Biochemistry 10, 3614–3621.

[14] Bechtel, P. J., Beavo, J. A. and Krebs, E. G. (1977) J. Biol. Chem. 252, 2691–2697.

[15] Peters, K. A., Demaille, J. G. and Fischer, E. H. (1977) Biochemistry 16, 5691–5697.

[16] Lee, P. C., Radloff, D., Schweppe, J. S. and Jungmann, R. A. (1976) J. Biol. Chem. 251, 914–921.

[17] Fuller, D. J. M., Byus, C. V. and Russel, D. H. (1978) Proc. Natl. Acad. Sci. USA 75, 223–227.

[18] Delbauffe, D., Ohayon, R. and Pavlovic-Hournac, M. (1979) Mol. Cell. Endocrinol. 14, 141–155.

[19] Pavlovic-Hournac, M., Delbauffe, D., Ohayon, R., Wadeleux, P. and Winand, R. (1979) FEBS Lett. 104, 21–24.

[20] Delbauffe, D. and Pavlovic-Hournac, M. (1976) FEBS Lett. 69, 59–62.

[21] Walsh, D. A., Ashby, C. D., Gonzales, C., Calkins, D., Fischer, E. A. and Krebs, E. G. (1971) J. Biol. Chem. 246, 1977–1985.

[22] Majumder, G. C. (1974) Biochem. Biophys. Res. Commun. 58, 756–762.

[23] Demaille, J. G., Peters, K. A. and Fischer, E. H. (1977) Biochemistry 16, 3080–3086.

[24] Alhanaty, E. and Shaltiel, S. (1979) Biochem. Biophys. Res. Commun. 89, 323–332.

[25] Friedman, Y., Lang, M., Levasseur, S. and Burke, G. (1979) Endocrinology 104, 467–475.

[26] Takasu, N., Charrier, B., Mauchamp, J. and Lissitzky, S. (1978) Eur. J. Biochem. 90, 139–146.