Biochimica et Biophysica Acta, 377 (1975) 271—281

BBA 67409

A CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE-DEPENDENT HISTONE KINASE FROM PIG BRAIN. PURIFICATION AND SOME PROPERTIES OF THE ENZYME

M.V. NESTEROVA, L.P. SASHCHENKO, V.Yu. VASILIEV and E.S. SEVERIN*

Institute of Molecular Biology, Academy of Sciences, Moscow (U.S.S.R.)

(Received June 10th, 1974)
(Revised manuscript received October 9th, 1974)

Summary

A cyclic adenosine 3′,5′-monophosphate-dependent histone kinase (ATP: protein phosphotransferase, EC 2.7.1.37) was isolated from pig brain. The enzyme has been purified 1140-fold; it is homogeneous on polyacrylamide gel electrophoresis and gel filtration. The estimated molecular weight of the enzyme is 120 000. Histone kinase dissociates into a catalytic subunit and a regulatory one (molecular weights 40 000 and 90 000, respectively). The catalytic subunit has been obtained in homogeneous state as evidenced by sodium dodecylsulphate-polyacrylamide gel electrophoresis. At all purification steps, enzymatic activity is stimulated 5-fold by cyclic AMP. An apparent K_m value for cyclic AMP is about $3.3 \cdot 10^{-7}$ M. In the presence of cyclic AMP ($5 \cdot 10^{-6}$ M), the K_m value for ATP and F1 histone were $1.2 \cdot 10^{-5}$ and $3 \cdot 10^{-5}$ M, respectively. Optimum pH value for histone kinase is 6.5, its isoelectric point is situated at pH 4.6. The purified enzyme displays high specificity for the lysine-rich and moderately lysine-rich histones F1, F2a2 and F2b. Arginine-rich histones and other known protein substrates for cyclic AMP-dependent protein kinases (casein, *Escherichia coli* RNA polymerase, etc.) are extremely poor substrates for this enzyme.

Introduction

In recent years a hypothesis of Kuo and Greengard [1] has been widely accepted, according to which the majority of cyclic AMP effects, or all of them, are based on the stimulation of enzymes catalysing phosphate transfer to

* To whom inquiries should be sent.
Abbreviation: EGTA, ethylene glycol bisaminoethyl ester of tetraacetic acid.

different protein substrates. Phosphorylation has been reported of different proteins such as casein, protamine, histones and some others, by partially purified enzyme preparations obtained from bacterial cells [1], skeletal muscle [2], liver [3], brain [4], heart [5] and red blood cells of mammals [6]. As a rule, cyclic AMP-dependent protein kinases (ATP:protein phosphotransferases, EC 2.7.1.37) consist of two types of subunits: one binding cyclic AMP, and the other one effecting γ-phosphate transfer from ATP to a protein. They are referred to as regulatory and catalytic subunits, respectively. The essence of enzyme activation by cyclic AMP is considered as due to binding of the nucleotide to the regulatory component of the protein kinase and ensuring dissociation of the oligomer resulting in release of an activated catalytic subunit [7—9]. A wide variety of effects is thus controlled by changes in the intracellular cyclic AMP content [10—12].

It is evident that the elucidation of the intimate mechanisms of protein kinase activation and of the nature of interaction between cyclic AMP and its receptor proteins represents an important problem. To study the cyclic AMP-sensitive enzymes in detail, the first essential step is to obtain homogeneous enzyme preparations. In this paper a purification procedure is described for the cyclic AMP-dependent histone kinase from pig brain catalysing phosphorylation of lysine-rich histones (F1, F2a2, F2b) and, to a lesser extent, of protamine. Some physical-chemical properties of the enzyme and data concerning its substrate specificity are reported.

Materials and Methods

Pig brain used as a source of the enzyme was stored in a refrigerator at −30 °C. Histone fractions were obtained from calf thymus by the method of Johns and Butler [13] and purified on CM-cellulose columns [14].

Cyclic AMP was purchased from Sigma, [γ-^{32}P]ATP and cyclic [^{3}H]AMP from Amersham, Sephadex from Pharmacia, DE-32 cellulose from Whatman. Hydroxyapatite was prepared by the procedure of Bernardi [15].

Histone kinase assay. Phosphotransferase activity (or kinase activity) of cyclic AMP-dependent histone kinase was measured by assaying ^{32}P incorporation into the histone F1. Incubation mixture (total volume 0.2 ml) contained 50 mM Tris · HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 mM EGTA, 2 mM theophylline, 1 mg/ml histone F1; 0.025 mM [γ-^{32}P]ATP (0.1 μCi/nmol), 5 μM cyclic AMP. Incubations were performed at 30 °C for 15 min. Reactions were stopped by cooling rapidly down to +5 °C, thereupon aliquots (0.1 ml) sampled from the reaction mixtures and applied to standard filter-paper discs (Whatman 3MM, diameter 24 mm). Filters were washed with 10% thrichloroacetic acid containing 10 mM sodium pyrophosphate, 10 mM sodium phosphate and 10 mM sodium tungstate for 30 min and three times by 5% trichloroacetic acid with the same ingredients. Washing was carried out at +5 °C. 15 ml trichloroacetic acid was used for each filter. After this procedure the filters were immersed into acetone/ethanol mixture (1 : 1, v/v) and into acetone, for 5 min each. Radioactivity of the dried filters was measured in a liquid-scintillation spectrometer SL-40 (Intertechnique). Scintillation liquid contained 4 g PPO and 200 mg POPOP per 1 l toluene. The specific activity of

the enzyme is represented as the number of ^{32}P nmol incorporated into the histone F1 per min by 1 mg enzyme under standard conditions of the phosphotransferase assay.

Assay of cyclic AMP-binding protein. The enzymatic activity in cyclic AMP binding was measured by the Gilman procedure [16] with minor modifications. The incubation mixture (total volume 0.4 ml) contained 50 mM acetate buffer (pH 4.0), 4 mM theophylline, 8 mM $MgCl_2$, 0.15 μM cyclic [^{3}H]AMP (1 μCi/nmol). Afer incubation at +4°C for 50 min the reaction was stopped by addition of 2 ml of 20 mM potassium phosphate buffer (pH 6.0), and the mixture was passed through Millipore filters HAWP. Each filter was washed with 15 ml of the buffer solution just mentioned. Radioactivity of the dried filters was measured as described above.

Isoelectric focusing. The isoelectric point of histone kinase was estimated by the isoelectric fucusing technique [17,18]. For this purpose the 110-ml column (LKB Instruments) was used containing 0—40% sucrose gradient and a mixture of ampholines providing a pH range of 3 to 10. Separation was carried out for 40 h at 4°C and voltage 500 V.

Polyacrylamide gel electrophoresis. A standard procedure described by Ornstein [19] and Davis [20] was applied. Samples of 50—100 μl volume containing 10—50 μg protein and 20% sucrose were layered on tubes with 5% gel 70 mm high. The run was carried out at a current of 2 mA per tube for 2 h. Gels were stained with Coomassie brilliant blue for 1 h and washed with methanol/acetic acid/water mixture (50 : 5 : 150, by vol.). The stained gels were analysed in a Chromoscan (Joyce-Loebl) at wavelength 620 nm. To measure enzymatic activity in the gel, gel cylinders were cut into slices 1 mm thick and dispersed in 0.2 ml 10 mM Tris · HCl buffer (pH 7.4) containing 4 mM mercaptoethanol, 2 mM EDTA and 200 mM NaCl. The protein eluted by this procedure was tested for activity by the standard assay, 20-μl aliquots were taken for this purpose.

Molecular weights were estimated by sodium dodecylsulphate-polyacrylamide gel electrophoresis as described by Shapiro et al. [21], with minor modifications. Runs were carried out in 0.1 M Tris · HCl buffer (pH 7.5) containing 0.1% sodium dodecylsulphate; a 7% gel was used.

Protein concentration was estimated by the procedure of Lowry et al. [22], or by ultraviolet spectrophotometry.

Results

Enzyme purification

All steps were carried out at 4°C; all solutions contained 2 mM EDTA and 4 mM mercaptoethanol.

Step 1. Homogenization. Frozen pig brain was thawed overnight at 4°C and homogenized in a Waring blender for 4 min at high speed. For 4 kg tissue, 10 l 10 mM Tris · HCl buffer (pH 6.8) was used. The homogenate was centrifuged for 1 h at 4500 × *g*.

Step 2. Acid precipitation. The supernatant was adjusted to pH 5.2 by dropwise addition of 1 M acetic acid with stirring, and centrifuged for 40 min at 4500 × *g*.

Step 3. DEAE-Sephadex A-50 chromatography. Supernatant was adjusted to pH 7.4, supplied with NaCl to a final concentration of 200 mM, and applied to a DEAE-Sephadex A-50 column (9 cm × 14 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.4) containing 200 mM NaCl. One column was loaded with 3 l supernatant. The elution of the histone kinase was achieved by passing 10 mM Tris · HCl (pH 7.4) containing 400 mM NaCl at a rate of about 1 l/h.

Step 4. $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the eluate from the Sephadex A-50 column up to 0.35 saturation. The mixture was kept for 1 h and centrifuged 30 min at 4500 × *g*. A new portion of $(NH_4)_2SO_4$ was added to the resulting supernatant to 0.60 saturation; the mixture left for 1 h and centrifuged 30 min at 16 000 × *g*. The precipitate was suspended in 50 mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl and dialysed overnight against the same buffer.

Step 5. Chromatography on hydroxyapatite. Dialysed enzyme preparation containing 750 mg protein was applied to a hydroxyapatite column (2 cm × 6 cm) equilibrated with 50 mM phosphate buffer (pH 6.8) containing 100 mM NaCl. After application of the protein the column was washed with the initial buffer. To remove the bulk of proteins, the column was then washed with 75 mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl. Histone kinase was eluted by 175 mM sodium phosphate buffer (pH 6.8) containing 100 mM NaCl at a flow rate of 60 ml/h. Protein was concentrated by precipitation with solid $(NH_4)_2SO_4$ up to 0.70 saturation.

Step 6. Gel filtration on Sephadex G-200. The enzyme preparation obtained at Step 5 was applied to a Sephadex G-200 column (2.5 cm × 90 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.4) containing 200 mM NaCl. Elution was achieved by the same buffer at a flow rate 12 ml/h, 3-ml fractions were collected. Fractions exhibiting histone kinase activity were combined and concentrated by ultrafiltration, using a XM-100 Amicon filter. Thereafter the protein solution was dialysed overnight against 10 mM Tris · HCl buffer (pH 7.4) containing 180 mM NaCl.

Step 7. DEAE-cellulose chromatography. The dialysed enzyme preparation was applied to a DE-32 cellulose column (1 cm × 50 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.4) containing 180 mM NaCl. The protein was eluted with the same buffer at a flow rate of 6 ml/h, 2-ml fractions were collected. A single symmetrical protein peak was eluted which coincided with the histone kinase activity peak and cyclic AMP-binding activity.

Comments on purification procedure. Typical results of the enzyme purification are presented in Table I. One can see that in the course of purification protein kinases and cyclic AMP-binding proteins are enriched differently. Owing to this, at the last steps a constant ratio is maintained between the capacity of the catalytic subunit to phosphorylate histone F1 and the capacity of the regulatory subunit to bind cyclic AMP. The described procedure makes it possible to obtain 30% yield of total activity and a 1140-fold purification.

Electrophoresis of the purified enzyme. When subjected to polyacrylamide gel electrophoresis 30 μg purified enzyme shows a single band on protein staining (Fig. 1A) and a single peak (Fig. 1B) on activity where the distribution patterns of histone kinase and cyclic AMP-binding capacity coincide.

Estimation of the enzyme molecular weight. Molecular weight was esti-

TABLE I

PURIFICATION OF HISTONE KINASE FROM PIG BRAIN

No.	Step	Protein (mg)	Activity (units)		Specific activity (units/mg)		Purification (-fold)	Recovery of activity	Correlation between catalytic and binding activity
			Catalytic	Binding	Catalytic	Binding			
1	Crude extract	42000	17388	58.8	0.414	0.0014	1	100	296
2	pH 5.2 supernatant	15000	15600	126	1.04	0.0084	2.5	90	124
3	DEAE-sephadex eluate	1500	14100	37.5	9.4	0.025	22.6	82	378
4	$(NH_4)_2SO_4$ precipitate	750	13500	35.5	18	0.048	43.5	78	374
5	Hydroxyapatite eluate	125	6750	24	54	0.19	130	39	284
6	Sephadex G-200 eluate	42	6600	23	154	0.55	370	38	276
7	DEAE-cellulose eluate	12	5600	20	470	1.65	1140	32	286

mated by gel filtration on a Sephadex G-200 column (1 cm × 52 cm) equilibrated with 10 mM Tris · HCl buffer (pH 7.4) containing 200 mM NaCl. Proteins were applied in a volume of 0.5 ml and eluted with the same buffer at a flow rate of 2.4 ml/h, 0.6-ml fractions were collected. For calibration the

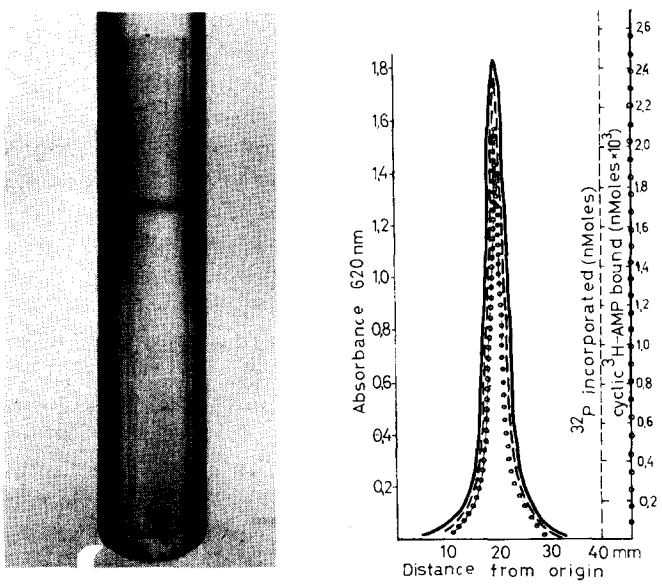


Fig. 1. Polyacrylamide gel electrophoresis of histone kinase. (A) After the run, one of the tubes was stained with Coomassie brilliant blue. (B) The stained tube was scanned at 620 nm (———). Another gel cylinder was cut into slices, and the protein was eluted as described in Materials and Methods. In the eluate histone kinase activity (- - - - -) and cyclic AMP-binding activity (— · — ·) were measured.

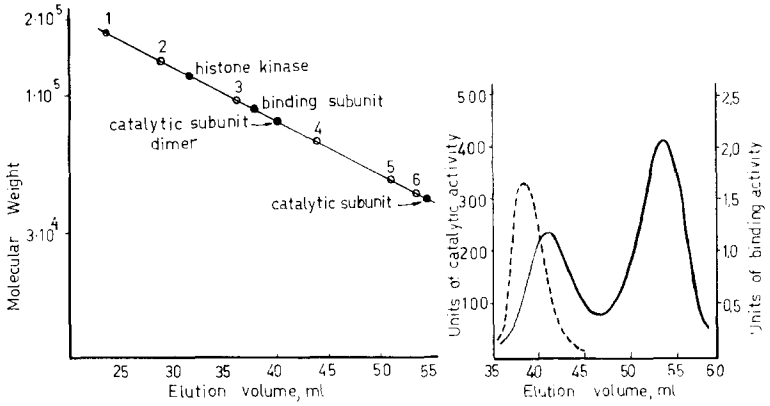


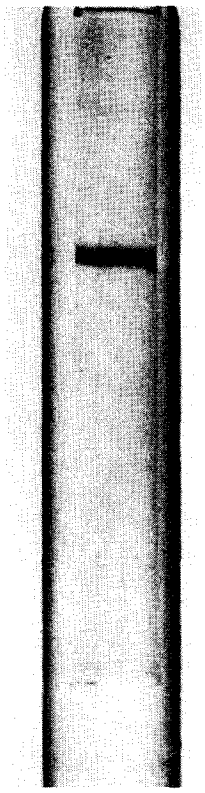
Fig. 2. (A) Calibration curve for the evaluation of molecular weight by gel filtration on Sephadex G-200. The column was calibrated with the following proteins: 1, rabbit muscle phosphorylase B (molecular weight 180 000); 2, bovine serum albumin, dimer (molecular weight 135 000); 3, pig heart aspartate transaminase (molecular weight 96 000); 4, bovine serum albumin, monomer (molecular weight 67 000); 5, rabbit muscle creatine kinase (molecular weight 47 000); 6, ovalbumin (molecular weight 42 000). (B) Gel filtration of histone kinase dissociating into subunits on a Sephadex G-200 column. Elution was carried out as described in the text. Cyclic AMP-independent histone kinase activity (———) and cyclic AMP-binding activity (- - - - -) were measured as described in Materials and Methods.

following proteins were used: rabbit muscle phosphorylase B, bovine serum albumin, aspartate transaminase from pig heart, rabbit muscle creatine kinase, ovalbumin. Elution volume of the kinase activity and cyclic AMP-binding activity corresponded to a molecular weight value of 120 000 (Fig. 2A).

Dissociation of the enzyme and estimation of molecular weight of the subunits by gel filtration

The enzyme was dialysed overnight at 4 °C against 10 mM Tris · HCl buffer (pH 7.4) containing 200 mM NaCl and 20 μM cyclic AMP. The enzyme solution was then applied to a Sephadex G-200 column (1 cm × 52 cm) equilibrated with the same buffer. Elution was carried out at a flow rate of 2.4 ml/h, 0.6-ml fractions were collected. The eluate was devoid of cyclic AMP-dependent histone kinase activity. Cyclic AMP-independent histone kinase activity was revealed in two peaks, whereas cyclic AMP-binding ability showed up in a single peak (Fig. 2B). The elution volume of the cyclic AMP-binding activity corresponds to a molecular weight of 90 000 whereas those of the peaks exhibiting cyclic AMP-independent histone kinase activity to the values 80 000 and 40 000 (Fig. 2A). These data indicate that the catalytic subunits can associate to form a dimer.

Isolation of the catalytic subunit. The enzyme obtained after Step 5 was dialysed overnight against 10 mM Tris · HCl buffer (pH 6.8) containing 50 mM NaCl and 20 μM cyclic AMP. The solution was applied to a DE-32 cellulose column (1 cm × 10 cm) equilibrated with the same buffer. The catalytic subunit was eluted with the same buffer at a flow rate 60 ml/h; volume of the fractions collected was 1.5 ml. A symmetrical protein peak was obtained coinciding with the peak of the cyclic AMP-independent histone kinase activity. No ability to bind cyclic AMP was revealed in this peak. Further elution with a linear salt gradient (50—400 mM NaCl) yielded proteins exhibiting cyclic AMP-



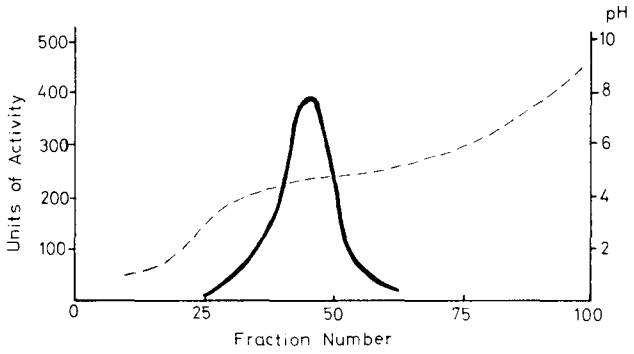


Fig. 3. Sodium dodecylsulphate-polyacrylamide gel electrophoresis of the catalytic subunit. The catalytic subunit preparation was treated with 1% sodium dodecylsulphate and 0.1 M mercaptoethanol at 37 °C before the run. Electrophoresis was carried out as described in the text.

Fig. 4. Isoelectric focusing of histone kinase. For experimental conditions, see Materials and Methods. - - - - - -, pH values; ———, histone kinase activity.

binding activity. The molecular weight of the catalytic subunit obtained by this procedure was estimated by means of sodium dodecylsulphate gel electrophoresis. The following proteins were used for calibration: bovine serum albumin (monomer, 67 000), heavy and light chains of γ-globulin (50 000 and 20 000, respectively), aspartate transaminase from pig heart (monomer 48 000), ovalbumin (42 000). On analysis by sodium dodecylsulphate gel electrophoresis, the catalytic subunit gave a single band (Fig. 3) of relative mobility corresponding to a molecular weight of 40 000.

Properties of the histone kinase

(1) Temperature stability of histone kinase. The histone kinase preparation was stored at −5° C for 1 month; the storage did not lead to any loss of enzymatic activity. After 10 min incubation of the enzyme at 50 °C at a concentration of 1 mg/ml without stabilizing agents, enzymatic activity decreased 2.6-fold, 1 h incubation resulted in 5.5-fold fall of activity. The highest stabilizing effect was displayed by mercaptoethanol (4 mM) and EDTA (2 mM).

(2) Isoelectric focusing. A typical pattern of isoelectric focusing of the

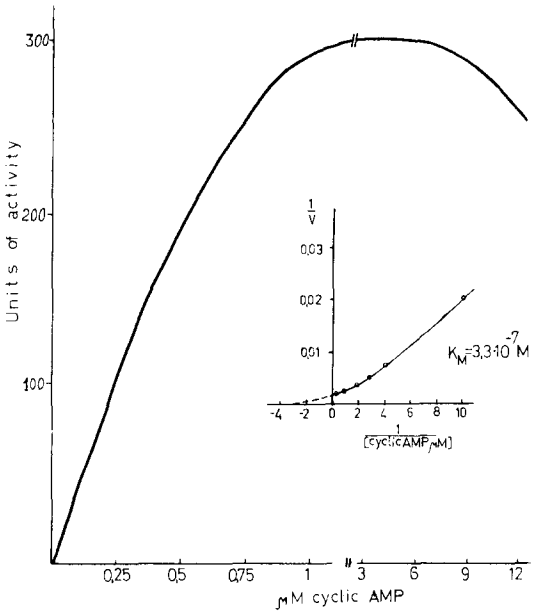


Fig. 5. Activity of histone kinase as a function of cyclic AMP concentration. The activity was assayed in 50 mM Tris · HCl buffer (pH 7.4) at a final concentration of histone F1 equal to 1 mg/ml. Values were corrected for 80 units of activity observed in the absence of added cyclic AMP.

enzyme is shown in Fig. 4. A cyclic AMP-dependent histone kinase was shown to have an isoelectric point at pH 4.6.

(3) pH dependence. The optimum pH value for histone kinase activity estimated in phosphate buffer was 6.5.

(4) Dependence on cyclic AMP concentration. At all purification stages the activity of histone kinase increased about 5-fold upon addition of cyclic AMP at a concentration of $2.5 \cdot 10^{-6}$ M. A plot of histone kinase activity versus cyclic AMP concentration is presented in Fig. 5. The concentration of cyclic AMP at which the activation of phosphorylation reaches one half of the maximum activation was approximately $3.3 \cdot 10^{-7}$ M. At cyclic AMP concentrations exceeding $6 \cdot 10^{-6}$ M a decrease of the stimulation effect is observed. Such dependence of the phosphorylation degree upon the cyclic AMP concentration (Fig. 5) may indicate the competition between cyclic AMP and ATP for binding in the active site of the catalytic subunit.

(5) Estimation of Michaelis constants for the substrates. K_m for ATP estimated at the cyclic AMP concentration ($5 \cdot 10^{-6}$ M) was $1.2 \cdot 10^{-5}$ M.

An apparent K_m value for histone F1 estimated under similar conditions was found to be $3 \cdot 10^{-5}$ M.

(6) Substrate specificity. Data concerning the substrate specificity of purified histone kinase are presented in Table II. One can see that the usual protein substrates of protein kinases such as serum albumin, casein, *Escherichia coli* RNA polymerase, protein from 30-S nuclear particles and some other proteins were completely incapable to be phosphorylated by the enzyme under study. Arginine-rich histones F2a1 and F3 did not accept phosphate residue to any significant extent. The isolated enzyme showed high substrate specificity with

TABLE II

PHOSPHORYLATION OF EXOGENOUS PROTEINS BY BRAIN KINASE

Protein substrate	Amount (μg)	Purified enzyme		Crude extract	
		– cyclic AMP	+ cyclic AMP	– cyclic AMP	+ cyclic AMP
Bovine serum albumin	200	0	0	0.3	0.3
Casein	200	0	0	0.3	0.3
Phosphorylase B	200	0	0	0.1	0.2
RNA polymerase (*E. coli*)	40	—	0	—	—
30-S nuclear particles protein	40	—	0	—	—
	200	—	4	—	—
Protamine	40	5	25		
	200	26	133	6	3
Histone F2a1	200	0	0	0.4	0.4
Histone F3	40	0	0		
	200	4.4	22	0.9	1.1
Histone F2a2	40	18	91		
	200	75	375	5.2	8
Histone F1	40	26	130		
	200	80	400	1.8	9
Histone F2b	40	71	355		
	200	85	425	7.4	17

respect to the lysine-rich histones F1, F2a2 and F2b. It is noteworthy that at early purification steps the specificity of the protein kinase was somewhat different: here histone F2b showed the highest phosphate acceptor capacity whereas that of histones F1 and F2a2 was somewhat lower. Crude enzyme preparation was able to phosphorylate serum albumin, casein, phosphorylase B and histones F2a1 and F3. Only when using histone F1 as the protein substrate throughout the purification procedure one can obtain highly purified histone kinase able to phosphorylate only the lysine-rich histones. Moreover, crude extract shows selective activation degrees with cyclic AMP for the phosphorylation of histones F1, F2a2 and F2b. Addition of cyclic AMP has almost no effect on the phosphorylation rate of serum albumin, casein and histone F2a1 catalysed by the crude extract. These data indicate that the cells of pig brain contain several protein kinases differing in substrate specificities. The lysine-rich histones being phosphorylated most actively.

Discussion

A great number of papers were devoted to the study of DNA-histone interaction and functional biological role of histones in the processes of chromatin activation [23—25]. In recent years ever increasing attention is being paid to reactions of specific modification of histones which are likely to notably affect functioning of the genetic apparatus [26—28]. In this respect the phosphorylation reaction is studied most extensively. This modification may control interaction between DNA and histones and may represent one of the main mechanisms regulating gene activity [29—31]. To evaluate the importance of histone phosphorylation for structural changes occurring in the chro-

matin during the cell cycle, it seems especially interesting to isolate and purify individual histone kinases catalysing selective phosphorylation of histones.

Several authors have described partial purification from different sources of various protein kinases, activated by cyclic AMP and showing wide substrate specificity [4,6,32]. Histones were shown to be the best acceptors of the phosphate moiety in reactions catalysed by these enzymes; however, other proteins also exhibited acceptor ability, e.g. ribosomal proteins [32], *E. coli* RNA polymerase [33], microtubulines [34], etc. Moreover, different histone fractions did not differ from each other in capacity to be phosphorylated. The observed lack of pronounced specificity of the phosphorylation reaction with regard to the protein substrates gave rise to various hypotheses trying to explain the broad substrate specificity of protein kinases [6,35].

Phosphorylation of histone F1 is supposed to be related to the chromosome compactization process and the structural state of chromatin at different stages of the cell cycle [26,36]. Therefore, we tried to purify a protein kinase which is specific for the lysine-rich histones. As mentioned above we succeeded in achieving this; the enzyme preparation obtained was found to be homogeneous on polyacrylamide gel disc electrophoresis and on gel filtration.

The data relating to substrate specificity of the cyclic AMP-dependent histone kinase indicate that the isolated enzyme may selectively recognize a certain amino acid sequence or pattern typical for the three lysine-rich histones (F1, F2a2, and F2b). Arginine-rich histones and other known substrates of the cyclic AMP-dependent protein kinases, casein, *E. coli* RNA polymerase, protein from the 30-S nuclear particles, fail to accept the phosphate moiety of ATP to any significant extent. From these data it can be inferred that the specific phosphorylation of lysine-rich histones, and in particular of histone F1, is catalysed by highly specific phosphokinases subject to control by cyclic AMP. Studies of dynamic aspects of the phosphorylation of different histones by highly specific histone kinases may be expected to provide insight into the nature of structural changes occurring in nucleoprotein complexes in the course of the interphase cycle and leading to condensation of the diffuse chromosomes before the onset of cell division.

References

1 Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 3417—3419
2 Walsh, D.A., Perkins, J.P. and Krebs, E.G. (1968) J. Biol. Chem. 243, 3763—3767
3 Langan, T.A. (1968) Science 162, 579—580
4 Miyamoto, E., Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395—6402
5 Rubin, C.S., Erlichman, J. and Rosen, O.M. (1972) J. Biol. Chem. 247, 36—44
6 Tao, M. and Hackett, P. (1973) J. Biol. Chem. 248, 5324—5332
7 Brostrom, C.O., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) Advances in Enzyme Regulation (Weber, G., ed.), Vol. 8, pp. 191—203, Pergamon Press, New York
8 Gill, G.N. and Garren, L.D. (1970) Biochem. Biophys. Res. Commun. 39, 335—343
9 Tao, M., Salas, M.L. and Lipmann, F. (1970) Proc. Natl. Acad. Sci. U.S. 67, 408—414
10 Corbin, J.D., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 4849—4851
11 Langan, T.A. (1973) Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A., eds), Vol. 3, pp. 99—153, Raven Press, New York
12 Schlender, K.K., Wei, S.H. and Villar-Palasi, C. (1969) Biochim. Biophys. Acta 191, 272—278
13 Johns, E.W. and Butler, J.A.V. (1962) Biochem. J. 82, 15—18
14 Johns, E.W. (1964) Biochem. J. 92, 55—59
15 Bernardi, G. (1971) Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds), Vol. 21, pp. 95—147, Academic Press, New York

16 Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305—312
17 Vesterberg, O. and Svensson, H. (1966) Acta Chem. Scand. 20, 820—834
18 Vesterberg, O., Wadström, T., Vesterberg, K., Svensson, H. and Malmgren, B. (1967) Biochim. Biophys. Acta 133, 435—445
19 Ornstein, L. (1964) Ann. N.Y. Acad. Sci. 121, 321—349
20 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
21 Shapiro, A.L., Viñuela, E. and Maizel J.V. (1967) Biochem. Biophys. Res. Commun. 28, 815—820
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
23 Balhorn, R., Bordwell, J., Sellers, L., Granner, D. and Chalkley, R. (1972) Biochem. Biophys. Res. Commun. 46, 1326—1333
24 Cross, M.E. (1972) Biochem. J. 128, 1213—1219
25 Kleinsmith, L.J., Allfrey, V.G. and Mirsky, A.E. (1966) Science 154, 780—781
26 Lake, R.S. (1973) J. Cell Biol. 58, 317—331
27 Lake, R.S., Goidl, J.A. and Salzman, N.P. (1972) Exp. Cell Res. 73, 113—121
28 Kleinsmith, L.J., Allfrey, V.G. and Mirsky, A.E. (1966) Proc. Natl. Acad. Sci. U.S. 55, 1182—1189
29 Gurley, L.R., Walters, R.A. and Tobey, R.A. (1973) Biochem. Biophys. Res. Commun. 50, 744—750
30 Louie, A.J., Sung, M.T. and Dixon, G.H. (1973) J. Biol. Chem. 248, 3335—3340
31 Louie, A.J. and Dixon, G.H. (1973) Biochem. Soc. Transaction 1, 682—684
32 Jergil, B. (1972) Eur. J. Biochem. 28, 546—554
33 Martelo, O.J., Woo, S.L.C., Reimann, E.M. and Davie, E.W. (1970) Biochemistry 9, 4807—4813
34 Goodman, D.B.P., Rasmussen, H., DiBella, F. and Guthrow, C.C.E. (1970) Proc. Natl. Acad. Sci. U.S. 67, 652—659
35 Lee-Jing Chen and Walsh, D.A. (1971) Biochemistry 10, 3614—3621
36 Gurley, L.R., Walters, R.A. and Tobey, R.A. (1974) J. Cell Biol. 60, 356—364