Biochimica et Biophysica Acta, 429 (1976) 798—808

BBA 67830

PROTEIN KINASES AND THEIR SUBSTRATES IN BROWN ADIPOSE TISSUE FROM NEWBORN RATS

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(Received October 27th, 1975)

Summary

The 100 000 × *g* supernatant fraction of brown fat from newborn rats catalyzed the cyclic AMP-dependent phosphorylation of both histone and a preparation of proteins from the same subcellular fraction (endogenous proteins). The apparent affinity for ATP was lower for the phosphorylation of the endogenous proteins than for the phosphorylation of histone. In order to discover whether the phosphorylation of histone and the endogenous proteins were catalyzed by different enzymes, the 100 000 × *g* supernatant was fractionated by ion-exchange and adsorption chromatography. Three different cyclic AMP-dependent protein kinases and one cyclic AMP-independent protein kinase were separated and partially purified. Each of these enzymes catalyzed the phosphorylation of both substrates, and the difference in apparent K_m for ATP remained. Neither affinity chromatography on histone-Sepharose, nor electrophoresis on polyacrylamide gels resulted in the separation of the phosphorylation of histone from that of the endogenous proteins of any of the partially purified kinases. Moreover, experiments in which the phosphorylated substrates were separated by differential precipitation with trichloroacetic acid showed that the endogenous proteins competitively inhibited the phosphorylation of lysine-rich histone. It is concluded that each of the partially purified kinase preparations contains protein kinase, which catalyzes the phosphorylation of both substrates. The difference in apparent K_m for ATP was found to be due to the presence in the endogenous protein preparation of a low molecular weight compound which competes with ATP. This was not ATP nor the modulator protein.

The ratio of the phosphorylation of endogenous proteins to that of histone was much higher for the cyclic AMP-independent kinase preparation than for the other enzymes. Electrophoresis of the endogenous substrates in the presence of sodium dodecyl sulphate showed that the enzyme phosphorylated a greater number of proteins than did the cyclic AMP-dependent kinases. The

phosphorylation of endogenous proteins relative to that of histone was significantly lower for one of the cyclic AMP-dependent kinases than for the other two. This difference was not reflected in a different pattern of phosphorylation of the individual proteins of the endogenous mixture.

Introduction

The major function of brown adipose tissue is to produce heat in response to the release of noradrenalin from sympathetic nerve endings within the tissue [1—3]. In rats, brown fat undergoes rapid proliferation and differentiation just before and after birth and during acclimatization of adult animals to cold. It becomes less active as the rat grows, or is returned to a warm environment [4, 5]. It is thought that the regulation of heat production, tissue proliferation and differentiation are all associated with the phosphorylation of specific proteins or enzymes [6—11]. Thus a study of brown fat of infant and cold-exposed rats offers a good opportunity to observe the relationship between protein kinases and their substrates under conditions in which the relative importance of the various physiological functions is changing. The activity of cyclic AMP-dependent protein kinase and the concentration of modulator proteins in brown fat are highest perinatally, and gradually decline as the animal grows [9,12]. We have recently isolated from brown fat of newborn rats a preparation of soluble proteins which acts as a substrate for cyclic AMP-dependent protein kinases [13]. The affinity of the protein kinase for ATP was lower with these endogenous proteins as substrate than with histone. Moreover, the phosphorylation of the endogenous protein with GTP as nucleotide substrate was greater than that of ATP. These observations suggested that brown fat of infant rats contained protein kinases with different affinities for protein substrates and for ATP [13]. This paper reports the separation and some of the properties of protein kinases of brown adipose tissue of newborn rats and their abilities to phosphorylate individual proteins in the endogenous preparation. It is also shown that the low affinity for ATP with the endogenous substrates is due to the presence in the preparation of a low molecular weight compound (not ATP) which competes with the ATP in the kinase assay.

Methods and Materials

Tissue extracts. Brown adipose tissue was obtained from the interscapular region of newborn Wistar rats. The tissue was homogenized in 2 vol. of ice-cold 0.25 M sucrose containing 1 mM EDTA and 10 mM Tris · HCl, pH 7.4, using a glass Potter-Elvehjem homogenizer fitted with a motor-driven Teflon pestle. The homogenate was centrifuged at 100 000 × *g* for 60 min in a Beckman L2-65B ultracentrifuge using an SW50.1 rotor at 4°C. The clear supernatant between the precipitate and the floating fat was used as the starting material for the separation of the protein kinases.

The supernatant containing endogenous substrates was obtained by homogenizing (as above) brown fat from newborn rats in 1 vol. of 4 mM EDTA, pH 7.0. After centrifugation at 100 000 × *g*, the clear supernatant was placed in a

boiling water bath for 1.5 min and then rapidly cooled in ice. Protein kinase modulator was purified from beef skeletal muscle by the method of Gilman [14].

Separation methods. Unless stated, the buffer used for chromatographic separations was 20 mM 2-(*N*-morpholino)ethane sulphonic acid, pH 6.5, containing 1 mM EDTA. The chromatographic media were equilibrated with buffer before use, and all chromatography performed at 4° C.

The 100 000 × *g* supernatant fraction was applied to a 20 × 1.6 cm column of DEAE-cellulose which was washed with 2 bed volumes of buffer. The column was eluted first with 0.1 M KCl and then with a linear gradient (0.1—0.5 M) of KCl. Fractions containing protein kinase activity were pooled within their separate peaks and dialysed at 4° C for 24 h against two changes of 20 volumes of buffer. The dialysed enzyme preparations were applied to 5 cm × 0.9 cm columns of phosphocellulose. The columns were then eluted with linear gradients (0—1.0 M) of KCl. Fractions containing protein kinase activity eluted by KCl were pooled and dialysed (as above). This dialysed enzyme and the kinase which was not retained on the column were then concentrated by ultrafiltration through a Pellicon (Type PS) membrane.

Affinity chromatography was performed on 10 × 0.9 cm columns of histone-Sepharose, which were eluted with linear gradients (0—0.5 M) of NaCl.

Chromatography on hydroxyapatite was performed in sodium phosphate buffer, pH 7.0, on 5 cm × 0.9 cm columns. After washing with 2 bed volumes of 5 mM sodium phosphate buffer, pH 7.0, the column was eluted with a linear gradient (5—200 mM) of sodium phosphate, pH 7.0.

Disc electrophoresis was performed on 7% polyacrylamide gels as described by Davis [15]. After electrophoresis the gels were sliced longitudinally and incubated with a protein substrate and [γ-^{32}P] ATP, as described by Hirsch and Rosen [16], for the detection of protein kinase activity directly on the gels. The gels were dried under vacuum and radioactivity detected by autoradiography using Kodirex X-ray film. Traces of the autoradiograms were obtained using a Joyce-Loebl densitometer. The method of Fairbanks et al. [17] was used for electrophoresis of phosphorylated proteins in the presence of sodium dodecyl sulphate. The gels were sliced longitudinally, dried under vacuum and autoradiographed.

Assays. Protein kinase activity was assayed as described previously, with histone (15 mg/ml) or the endogenous proteins (6 mg/ml) as the protein substrate [18]. With both substrates the rate of phosphorylation was a linear function of time for up to 10 min and of enzyme protein up to 50 μg per assay. The same conditions were used to phosphorylate endogenous proteins before electrophoresis in dodecyl sulphate, except that the specific activity and concentration of the [γ-^{32}P] ATP were approx. 6000 dpm/pmol and 67 μM, respectively, and the reaction was stopped by the addition of dodecyl sulphate (1.0% finally). The mixture was incubated at 37° C for 30 min in the presence of 0.1% dithiothreitol and 100 mM sodium phosphate before being subjected to electrophoresis. Apparent K_m values were determined from the intercepts of double-reciprocal plots of enzyme activity against substrate concentration. The slopes of the lines were calculated by the method of least squares.

Protein was assayed by the method of Lowry et al. [19].

Materials. [γ-^{32}P]ATP (10—15 Ci/nmol) was purchased from The Radiochemical Centre, Amersham, U.K. Non-labelled nucleotides, lysine-rich histone and histone (Type IIA) were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Histone-Sepharose was prepared as described by Corbin et al. [20]. DEAE-Cellulose (DE11) and phosphocellulose (P11) were from Whatman Biochemicals Ltd., Maidstone, Kent, U.K., and hydroxyapatite (Bio-Gel HTP) from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Pellicon (Type PS) ultrafiltration membranes were obtained from Millipore Corporation, Bedford, Mass., U.S.A., and Kodirex X-ray film from Kodak Ltd., London, U.K.

Results

Separation of protein kinases

Four major peaks of protein kinase activity were detected when the 100 000 $\times$ *g* supernatant fraction from brown fat of newborn rats was chromatographed on DEAE-cellulose (Fig. 1). The activities of Peaks I, IIA and IIB were not retained on a column of phosphocellulose, whereas that of Peak III was retained and eluted with approx. 0.75 M KCl. The activities obtained after phosphocellulose chromatography will be referred to as kinases I, IIA, IIB and III, after the DEAE-cellulose peaks from which they were derived. It was confirmed that kinases IIA and IIB were separate enzymes by chromatography on hydroxyapatite. Kinase IIA was eluted from hydroxyapatite by 0.15 M sodium phosphate, whereas kinase IIB was eluted by 0.05 M sodium phosphate.

Some of the properties exhibited by the enzymes obtained in a typical purification through DEAE-cellulose and phosphocellulose are shown in Table I. Each enzyme catalyzed the phosphorylation of both histone and the boiled preparation of endogenous proteins. All kinase activities except that of kinase III with endogenous proteins as substrates were stimulated by cyclic AMP and inhibited by the modulator protein. There was no significant difference between enzymes in the apparent K_m values for histone (approx. 3 mg/ml) or the endogenous proteins (approx. 2 mg/ml). However, the ratio of phosphorylation of endogenous proteins to phosphorylation of histone was different for each enzyme. Values obtained for four separate preparations of enzymes (mean $\pm$ S.E.) were 0.086 $\pm$ 0.008 for kinase I, 0.096 $\pm$ 0.015 for kinase IIA, 0.054 $\pm$ 0.007 for kinase IIB and 0.685 $\pm$ 0.063 for kinase III. Protein kinase activities of IIA and IIB were additive, so it is unlikely that the difference in ratio was due to a contamination of one of the enzymes with modulator proteins or phosphatases.

The apparent K_m for ATP (mean $\pm$ S.E. for five preparations) of the phosphorylation catalyzed by the 100 000 $\times$ *g* supernatant fraction was 0.045 $\pm$ 0.008 mM with histone as substrate and 0.186 $\pm$ 0.061 mM with endogenous proteins as substrate. There was no significant difference between the partially purified kinases in apparent K_m values for ATP. However, for each kinase preparation the apparent K_m for ATP with histone as substrate (approx. 0.012 mM) was still lower than that with endogenous proteins as substrate (approx. 0.065 mM). Since the original explanation for this difference in apparent K_m for ATP was that the phosphorylation of the two substrates was catalyzed by different enzymes [13], further attempts to separate activity which phosphorylated his-

tone from that which phosphorylated the endogenous proteins were made by subjecting the kinases to affinity chromatography and disc-gel electrophoresis.

Kinases IIA and IIB were mixed and applied to a column of histone-Sepharose. All protein kinase activity was retained on the column and was eluted with a linear gradient of NaCl. The elution of the activity which phosphorylated endogenous proteins was very similar to that of the activity which phosphorylated histone.

The impure phosphocellulose eluates were also subjected to disc electrophoresis on polyacrylamide gels. The kinase bands were detected directly on the gels as described in Methods and Materials. Typical densitometer traces of the autoradiograms are shown in Fig. 2. Values of R_F are given as mean ± S.E. of three separate runs. The histone kinase activity of kinase I migrated as a single band with an R_F on 0.247 ± 0.004 (Fig. 2A) and that of kinase IIA as a single band with an R_F of 0.286 ± 0.006 (Fig. 2C). The histone kinase of the preparation of kinase IIB used for electrophoresis (which was a different preparation from that applied to hydroxyapatite) migrated as two bands. The major band had an R_F of 0.243 ± 0.004 and the minor band (seen as a shoulder on the densitometer trace) an R_F of 0.285 ± 0.003 (Fig. 2B). The minor band probably resulted from an incomplete separation from kinase IIA during DEAE-cellulose chromatography. Kinase activity detected using endogenous proteins as substrates migrated with the same R_F values as the activity detected using histone (Figs. 2A and 2B). The activities of kinase III which catalyzed the phosphorylation of both histone and endogenous proteins only just penetrated the gels (Fig. 2D). The R_F value for both activities was 0.018 ± 0.001. Minor bands were observed in kinase I (R_F of 0.456 ± 0.006) and kinase III (R_F of approx. 0.2) when histone was used as substrate and in kinase I (R_F of 0.077 ± 0.004) when endogenous proteins were used as substrate.

In the light of these results, it was clear that the hypothesis that the phos-

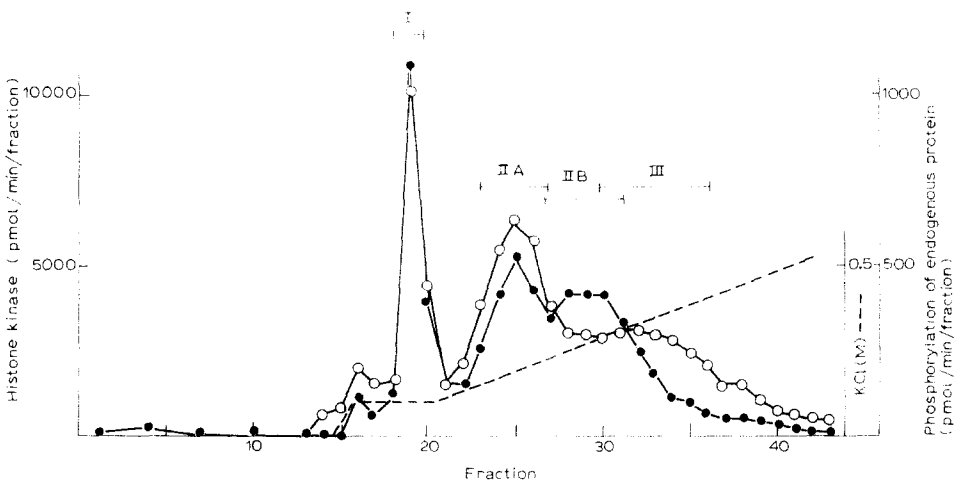

Fig. 1. The separation of protein kinase activities on DEAE-cellulose. The 100 000 × *g* supernatant fraction from brown fat was applied to a column (20 cm × 1.6 cm) of DEAE-cellulose. The column was eluted with 0.1 M KCl followed by a linear (0.1—0.5 M) gradient of KCl and 3.5-ml fractions were collected. Each fraction was assayed for protein kinase activity with histone (●) or endogenous proteins (○) as substrate in the presence of cyclic AMP (2 μM).

TABLE I

THE SPECIFIC ACTIVITY AND RECOVERY OF THE PARTIALLY PURIFIED PROTEIN KINASES AND THE EFFECT OF CYCLIC AMP (2 μM) AND MODULATOR PROTEIN (500 μg/ml) ON THE PHOSPHORYLATION OF HISTONE AND ENDOGENOUS PROTEINS

Recovery of protein kinase activity is given as a percentage of that in the 100 000 × *g* supernatant fraction. Inhibition by the modulator protein from beef muscle is expressed as a percentage of the uninhibited activity. The activity ratio is the ratio of the phosphorylation of endogenous proteins (6 mg/ml) to that of histone (15 mg/ml) in a 10-min assay with 0.2 mM [γ-^{32}P] ATP. Results are given for enzymes obtained during the same purification procedure.

Kinase preparation	Histone as substrate				Activity ratio endogenous proteins / Histone	Endogenous proteins as substrate			
	Spec. act. (pmol/min per mg protein)	Recovery (%)	+ Cyclic AMP / − Cyclic AMP	Inhibition by modulator		Spec. act. (pmol/min per mg protein)	Recovery (%)	+ Cyclic AMP / − Cyclic AMP	Inhibition by modulator (%)
100 000 × *g* supernantant	1 070	100	13.4	72	0.089	95	100	2.4	67
Kinase I	5 512	14	4.0	65	0.058	316	9	3.1	90
IIA	12 986	29	5.5	100	0.062	805	20	4.3	100
IIB	7 756	20	5.8	88	0.046	357	10	4.4	100
III	234	1	4.0	62	0.611	143	6	1.0	12

phorylation of histone and endogenous proteins were catalyzed by different enzymes would have to be revised. Ion-exchange chromatography had separated a cyclic AMP-independent protein kinase which phosphorylated endogenous proteins rather than histone (kinase III), but this did not account for all of the phosphorylation of endogenous proteins. The remainder (about 50%) was catalyzed by cyclic AMP-dependent protein kinases. Thus consideration had to be given to the possibility that the higher apparent K_m for ATP observed with the endogenous proteins as substrate was caused by some factor in the endogenous preparation and not by the presence of two separate enzymes.

In order to check whether some component in the endogenous substrate preparation was affecting the apparent K_m for ATP, it was necessary to separate the histone substrate from the endogenous proteins after phosphorylation. This was achieved by using lysine-rich histone, which is precipitated at a higher concentration of trichloroacetic acid than are the endogenous proteins or the mixed histone. At the end of the incubation some of the mixture was treated with 5% trichloroacetic acid, which precipitated all the endogenous proteins but only about 25% of the lysine-rich histone. An equal volume was treated with 20% trichloroacetic acid, which precipitated all of the lysine-rich histone. Thus, the difference between the radioactivity precipitated at 20 and 5% trichloroacetic acid represented only the phosphorylation of the histone. The results are shown in Fig. 3. The apparent K_m for ATP of kinase IIA was 0.013 mM with lysine-rich histone alone. In the presence of the preparation of endogenous proteins, the apparent K_m for ATP of the phosphorylation of the histone (0.055 mM) was similar to that of the phosphorylation of the endogenous proteins alone (0.057 mM). The same procedure was used to show that the endogenous proteins competitively inhibited the phosphorylation of histone. The apparent K_m of kinase IIA for lysine-rich histone was 4.1 mg/ml in the absence, and 6.6 mg/ml in the presence, of endogenous proteins (6 mg/ml).

Clearly some component of the endogenous protein preparation was either competing directly with ATP or was affecting the catalytic site of the enzyme. The preparation was filtered and passed through a column (20 × 0.9 cm) of Sephadex G-150. Protein substrates were eluted near the void volume. The apparent K_m for ATP using these proteins as substrates was the same as that found with histone (0.015 mM). The compounds which affected the K_m for ATP were eluted from the Sephadex in approximately three times the void volume, which corresponded to a molecular weight of under 5000. This fraction was well separated from the modulator protein (which did not affect the K_m for ATP) and exhibited an inhibition which was competitive with ATP. This inhibition was not lost after treatment with trypsin or an ATPase (glycerol plus glycerokinase).

Separation of endogenous substrates

There were differences between the four isolated kinases in the ratio of the phosphorylation of endogenous proteins to that of histone. To check whether the kinases phosphorylated different proteins in the endogenous mixture, portions of the endogenous protein preparation were incubated with [γ-^{32}P] ATP and the various enzyme preparations. The reactions were stopped by the addition of sodium dodecyl sulphate and the proteins separated by electrophoresis in

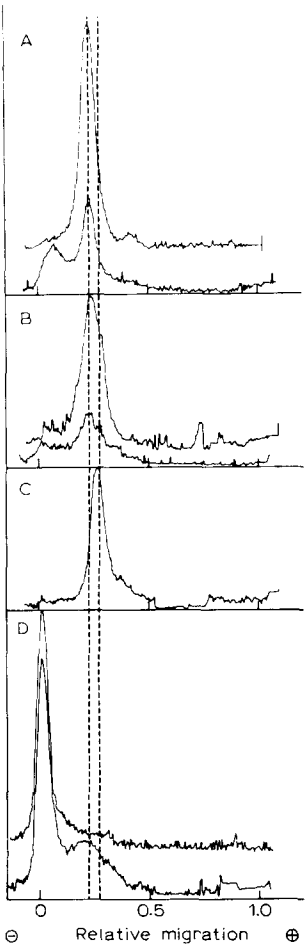

Fig. 2. Densitometer traces of autoradiograms of protein kinase activity detected on polyacrylamide gels after disc electrophoresis. The enzyme preparations (0.2 ml per gel) were subjected to electrophoresis on 7% polyacrylamide gels. After electrophoresis the gels were sliced longitudinally. Protein kinase activity was detected directly on the gels using 50 μM [γ-^{32}P] ATP (specific activity approx. 200 cpm/pmol) and either histone or endogenous proteins as substrates (see Methods and Materials). The gels were then dried and radioactivity detected by autoradiography. (A) Kinase I detected with histone (upper trace) and endogenous proteins (lower trace). (B) Kinase IIB detected with histone (upper trace) and endogenous proteins (lower trace). (C) Kinase IIA detected with histone. (D) Kinase III detected with endogenous proteins (upper trace) and histone (lower trace). Each pair of traces was obtained with the opposite halves of the same gels.

the presence of dodecyl sulphate as described in Methods and Materials. Kinase I, IIA and IIB generally phosphorylated the same proteins. Three major radioactive bands were present, with R_F values of 0.02, 0.48 and 0.70 (Fig. 4). Minor bands could be detected with R_F values of 0.13, 0.30, 0.34, 0.41, 0.56 and 0.63. The phosphorylation of each of these proteins was stimulated by cyclic AMP (Fig. 4C). There were small differences between the enzymes in the relative degree of phosphorylation of the bands with R_F values of 0.02, 0.13 and 0.70. Only one phosphorylated band (R_F 0.48) of relatively low intensity was present if the enzymes were incubated with [γ-^{32}P] ATP in the absence of endogenous proteins (Fig. 4B).

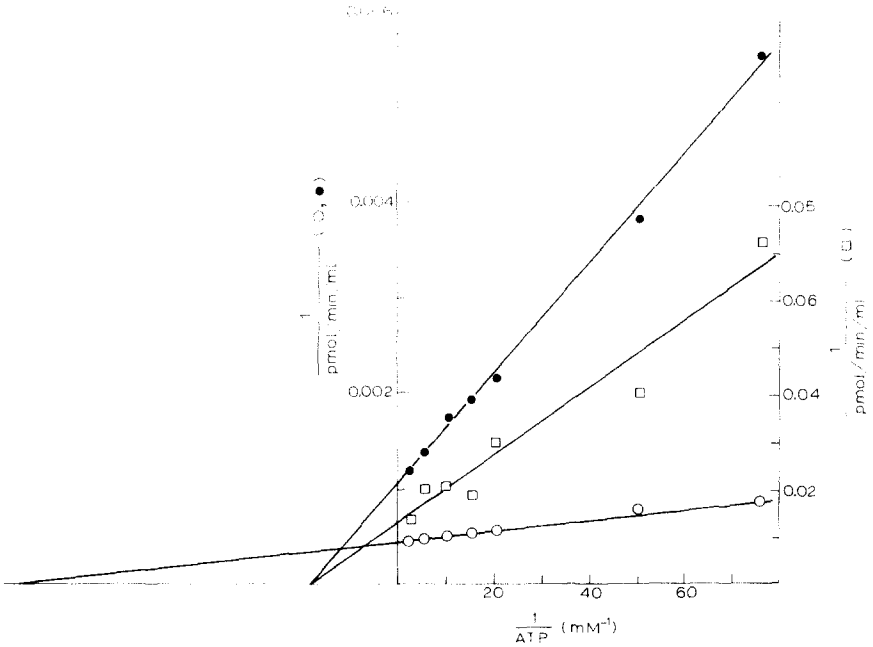

Fig. 3. Double-reciprocal plots of the effect of the concentration of ATP on the phosphorylation of endogenous proteins (□), and lysine-rich histone in the presence (●) and absence (○) of endogenous proteins. Phosphorylation of lysine-rich histone (15 mg/ml) in the presence of endogenous (6 mg/ml) was measured after differential precipitation of the phosphorylated protein mixture with trichloroacetic acid (see text for details).

Kinase III phosphorylated a far greater range of proteins than kinases I and II (Fig. 4D). Cyclic AMP did not affect the phosphorylation of any of these proteins. Radioactive bands were found in each of the positions occupied by the proteins phosphorylated by kinases I and II. In addition there were major bands with R_F values of 0.26, 0.51 (which appears on the densitometer trace as a shoulder on the 0.48 peak) and 0.58. Kinase III also phosphorylated three proteins of low molecular weight (R_F values of 0.77, 0.85 and 0.89).

Discussion

Previous experiments with the 100 000 × *g* supernatant fraction of brown fat from newborn rats suggested that the cyclic AMP-dependent phosphorylation of histone and the endogenous protein preparation were catalyzed by different enzymes [13]. However, the present work shows that the simple separation that was expected was not achieved. Each of the partially purified enzymes catalyzed the phosphorylation of both histone and the endogenous substrates. Although the catalytic activities towards histone and the endogenous proteins were eluted together from affinity chromatography on histone-Sepharose, the degree of purification was still too low to demonstrate conclusively that each enzyme phosphorylated the two substrates at the same catalytic site. Because of the small amount of starting material, and the instability of the enzymes (particularly kinase IIA), it was not possible to subject the kinases eluted from

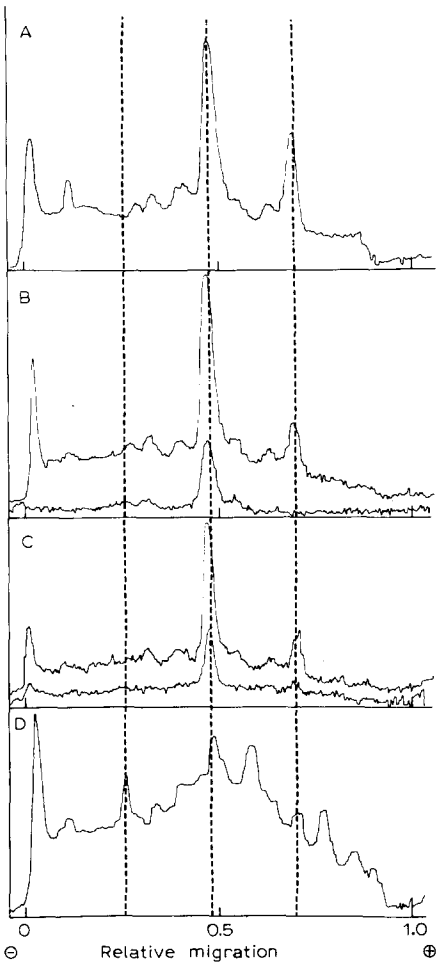

Fig. 4. Densitometer traces of autoradiograms of the radioactive bands obtained from dodecyl sulphate electrophoresis of the endogenous proteins after phosphorylation with [γ-^{32}P] ATP. The endogenous proteins were phosphorylated at 30°C for 5 min with 67 μM [γ-^{32}P] ATP (specific activity approx. 6000 cpm/pmol) as substrate. The reaction was stopped and the proteins dissociated as described in Methods and Materials. The phosphorylated proteins (0.03 ml of reaction mixture) were separated by electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulphate. The gels were then sliced longitudinally, dried and autoradiographed (see Methods and Materials for details). (A) Phosphorylation catalyzed by kinase I in the presence of cyclic AMP (2 μM). (B) Phosphorylation catalyzed by kinase IIB in the presence of cyclic AMP (upper trace) and in the presence of cyclic AMP, but with no endogenous substrates (lower trace). (C) Phosphorylation catalyzed by kinase IIA in the presence (upper trace) and absence (lower trace) of cyclic AMP. (D) Phosphorylation catalyzed by kinase III.

histone-Sepharose or hydroxyapatite to any further purification procedures. However, confirmation of the results obtained from chromatography was given by disc electrophoresis of the impure phosphocellulose eluates on polyacrylamide gels. For each enzyme, kinase activity detected using endogenous proteins migrated with an R_F value identical with that detected using histone.

Further evidence that both substrates were phosphorylated at the same catalytic site was obtained from experiments using lysine-rich histone, which indicated that the endogenous proteins competitively inhibited the phosphorylation of histone. The kinetic data should, however, be treated with some cau-

Acknowledgments

I wish to thank Mrs. R.A. Fordham for her excellent technical assistance, and Dr. N.B. Myant for his help and encouragement.

References

1 Smith, R.E. and Horwitz, B.A. (1969) Physiol. Rev. 49, 330—425
2 Hull, D. and Segall, M.M. (1965) J. Physiol. Lond. 181, 458—467
3 Knight, B.L. and Myant, N.B. (1970) Biochem. J. 119, 103—111
4 Barnard, T. and Skala, J. (1970) Brown Adipose Tissue (Lindberg, O., ed.), pp. 33—72, Elsevier, New York
5 Smith, R.E. and Roberts, J.C. (1964) Am. J. Physiol. 206, 143—148
6 Knight, B.L. (1974) Biochim. Biophys. Acta 343, 287—296
7 Knight, B.L. and Fordham, R.A. (1975) Biochim. Biophys. Acta 384, 102—111
8 Skala, J.P. and Hahn, P. (1974) Int. J. Biochem. 5, 95—106
9 Skala, J., Novak, E., Hahn, P. and Drummond, G.I. (1972) Int. J. Biochem. 3, 229—242
10 Langan, T.A. (1973) Adv. Cyclic Nucleotide Res. 3, 99—153
11 Wicks, W.D., Barnett, C.A. and McKibbin, J.B. (1974) Fed. Proc. Fed. Am. Soc. Exp. Biol. 33, 1105—1111
12 Skala, J.P., Drummond, G.I. and Hahn, P. (1974) Biochem. J. 138, 195—199
13 Knight, B.L. and Skala, J.P. (1975) Biochim. Biophys. Acta 385, 124—132
14 Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305—312
15 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
16 Hirsch, A. and Rosen, O.M. (1974) Anal. Biochem. 60, 389—394
17 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
18 Skala, J.P. and Knight, B.L. (1975) Biochim. Biophys. Acta 385, 114—123
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
20 Corbin, J.D., Brostrom, C.O., King, C.A. and Krebs, E.G. (1972) J. Biol. Chem. 247, 7790—7798
21 Erlichman, J., Rosenfeld, R. and Rosen, O.M. (1974) J. Biol. Chem. 249, 5000—5003
22 Maeno, H., Reyes, P.L., Ueda, T., Rudolph, S.A. and Greengard, P. (1974) Arch. Biochem. Biophys. 164, 551—559
23 Knight, B.L. (1975) Biochem. J. 152, 577—582
24 Miyamoto, E., Petzold, G.L., Kuo, J.F. and Greengard, P. (1973) J. Biol. Chem. 248, 179—189