PHOSPHORYLATION OF RABBIT SKELETAL MUSCLE PHOSPHORYLASE KINASE BY CYCLIC GMP-DEPENDENT PROTEIN KINASE

Philip COHEN

Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee DD1 4HN, Scotland

Received 21 August 1980

1. Introduction

Cyclic GMP-dependent protein kinase is a distinct enzyme from cyclic AMP-dependent protein kinase which may mediate the actions of cyclic GMP in eukaryotic cells [1]. It was suggested initially that its substrate specificity was quite different from cyclic AMP-dependent protein kinase, since it was reported to phosphorylate distinct tryptic peptides on mixed histones and to be unable to activate phosphorylase kinase [2–4]. Subsequently, however, many of the substrates for cyclic AMP-dependent protein kinase, including phosphorylase kinase, were found to be capable of phosphorylation by cyclic GMP-dependent protein kinase, although the rates of phosphorylation were 10–50-fold slower [5–11].

In this paper it is demonstrated that cyclic AMP- and cyclic GMP-dependent protein kinases phosphorylate the same two sites on phosphorylase kinase, but at quite different relative rates. The results suggest that cyclic GMP-dependent protein kinase may be of general use in elucidating the functions of specific phosphorylation sites in those enzymes that are phosphorylated at several positions by cyclic AMP-dependent protein kinase.

2. Materials and methods

2.1. *Materials*

[γ-^{32}P] ATP was purchased from the Radiochemical Centre, Sephadex G-50 (superfine grade) from Pharmacia, and *N'*-(2-hydroxyethyl)-ethylenediamine-*N*,*N*,*N'*-triacetate (HEDTA) and nitrilotriacetic acid (NTA) from Sigma.

2.2. *Protein preparations*

Phosphorylase kinase [12], phosphorylase *b* [13], the catalytic subunit of cyclic AMP-dependent protein kinase [14] and its specific protein inhibitor [15], protein phosphatase-1 [16], protein phosphatase-2 [17], and protein phosphatase inhibitor-2 [18], were purified from rabbit skeletal muscle by standard procedures. These proteins were provided by Mr F. B. Caudwell, Dr J. G. Foulkes, Dr P. Parker, Dr C. Picton and Mr A. A. Stewart in this laboratory. Cyclic GMP-dependent protein kinase was purified from bovine lung according to Walter et al. [19] except that the gel filtration step was omitted. This preparation, which was 70% pure, was provided by Dr D. Aswad and Professor P. Greengard, Yale University, USA. Trypsin treated with tosylphenylchloromethylketone was a product of Worthington Biochemicals, purchased from Cambrian Chemicals, Croydon, Surrey England.

2.3. *Assay of phosphorylase kinase*

Phosphorylase kinase was assayed as in [20,21] using a calcium HEDTA buffer at pH 6.8 and a calcium-NTA buffer at pH 8.2. The final concentrations of HEDTA, NTA and Ca^{2+} were 1.0 mM, 1.0 mM and 0.8 mM respectively, giving free Ca^{2+} concentrations of 0.06 and 0.07 mM at pH 6.8 and 8.2 respectively. These values are saturating for both the phosphorylated and dephosphorylated forms of the enzyme [21].

2.4. *Phosphorylation of phosphorylase kinase*

2.4.1. Cyclic AMP-dependent protein kinase

The incubations were carried out at 20°C and contained phosphorylase kinase 2.0 mg/ml, sodium

glycerophosphate 10 mM, EDTA 0.4 mM, EGTA 0.2 mM, magnesium acetate 2.0 mM, the catalytic subunit of cyclic AMP-dependent protein kinase and [γ-^{32}P]ATP 0.2 mM (10^8 cpm/μmole). The reactions were initiated with ATP and aliquots were removed at various times and assayed for phosphorylase kinase activity and for phosphorylation. For calculation of the stoichiometry of phosphorylation, the molecular weight was taken as 335 000 ($\alpha\beta\gamma\delta$ unit) and the absorbance index $A^{1\%}_{280\ nm}$, as 12.4 [12]. Further aliquots were denatured in sodium dodecyl sulphate and subjected to electrophoresis on 5% polyacrylamide gels. The gels were stained, destained, and the α, β, γ and δ-subunits cut out and analysed by Cerenkov counting.

2.4.2. Cyclic GMP-dependent protein kinase

The incubations were identical, except that cyclic GMP-dependent protein kinase replaced cyclic AMP-dependent protein kinase, and cyclic GMP (1.0 μM) and the specific protein inhibitor of cyclic AMP-dependent protein kinase were included. The protein kinase inhibitor was added because phosphorylase kinase is contaminated with traces of cyclic AMP-dependent protein kinase.

2.5. *Dephosphorylation of phosphorylase kinase*

Phosphorylase kinase (1.0 mg) was phosphorylated by cyclic AMP-dependent protein kinase (0.3 U/ml – see [15,22] for definition of units) as in section 2.4. After 10 min, when 1.7 mol of phosphate per $\alpha\beta\gamma\delta$ unit had been incorporated, the reaction was stopped by the addition of EDTA (10 mM). The solution was passed through Sephadex G-50 Superfine (10 × 1 cm) equilibrated in 50 mM Tris/HCl – 50 mM 2-mercaptoethanol pH 7.0. The [^{32}P]phosphorylase kinase (0.8 mg/ml), was then incubated at 20°C with either protein phosphatase-1 (5.8 U/ml) in the presence of 1.0 mM EDTA, or with protein phosphatase-2 (1.0 U/ml) in the presence of 1.0 mM $MnCl_2$ and protein phosphatase inhibitor-2 (85 U/ml), a specific inhibitor of protein phosphatase-1 [22]. Units of phosphatase activity are defined in [15,18]. Aliquots were withdrawn at various times and assayed for phosphorylase kinase, phosphate released, and phosphate remaining in the α and β-subunits.

3. Results

3.1. *Phosphorylation of phosphorylase kinase by cyclic AMP and by cyclic GMP-dependent protein kinases*

Cyclic GMP-dependent protein kinase was found to phosphorylate and activate phosphorylase kinase, confirming earlier reports [5,6]. However much less activation occurred than with cyclic AMP-dependent protein kinase for the same amount of phosphate incorporated (fig.1). The rate of activation by cyclic GMP-dependent protein kinase was 2.5–3-fold slower than that catalysed by cyclic AMP-dependent protein kinase, although the initial rate of phosphorylation was 1.3-fold faster. This indicated that the two enzymes did not phosphorylate the same site preferentially, an observation confirmed by investigation of the phosphorylation of the individual subunits of phosphorylase kinase. Cyclic GMP and cyclic AMP-dependent protein kinases both phosphorylated the α and β-subunits, but the relative rates of phosphorylation of these two components were quite different. Cyclic AMP-dependent protein kinase initially phosphorylated the β-subunit 5-fold faster than the α-subunit, whereas cyclic GMP-dependent protein kinase phosphorylated the α-subunit 3-fold faster than the β-subunit (fig.2). No phosphate was incorporated into the γ and δ-subunits, by either enzyme. There was an excellent correlation between the activity of phosphorylase kinase and the degree of phosphorylation of the β-subunit, and no correlation between activation and the degree of phosphorylation of the α-subunit (fig.3).

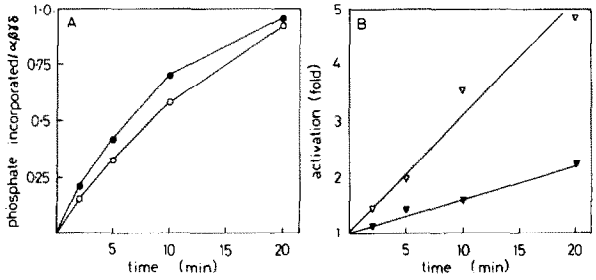

Fig.1. Phosphorylation (A) and activation (B) of phosphorylase kinase by cyclic GMP and cyclic AMP-dependent protein kinases. Phosphorylation is shown by circles and activation at pH 6.8 triangles. Closed symbols show results with cyclic GMP-dependent protein kinase and open symbols results with cyclic AMP-dependent protein kinase. In the absence of either protein kinase the incorporation of phosphate was <0.05 molecules per $\alpha\beta\gamma\delta$ unit after 20 min.

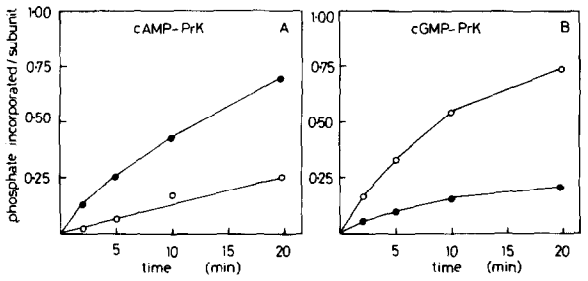

Fig.2. Phosphorylation of the α-subunit (open circles) and β-subunit (closed circles) of phosphorylase kinase by (A) cyclic AMP-dependent protein kinase (cAMP-PrK), and (B) cyclic GMP-dependent protein kinase (cGMP-PrK). No phosphate was incorporated into the γ or δ-subunit by either enzyme.

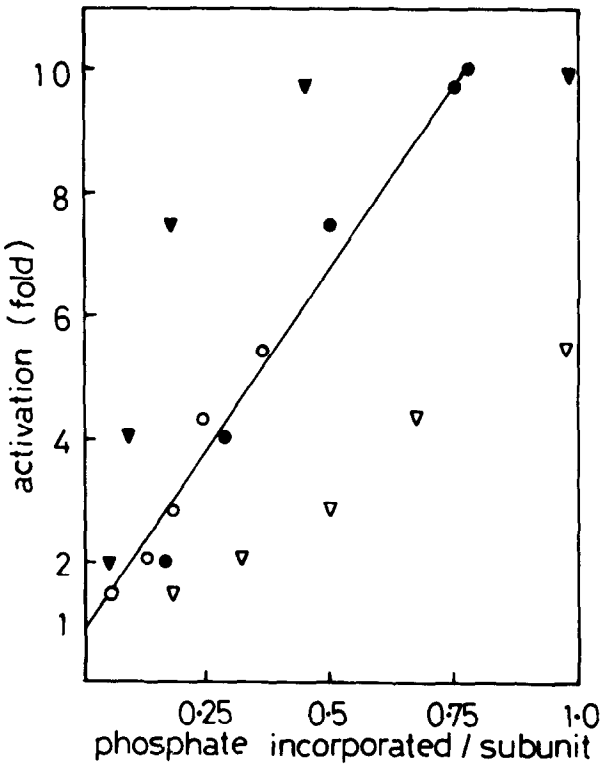

Fig.3. Correlation between activation of phosphorylase kinase and phosphorylation of the β-subunit. Phosphorylation of the β-subunit is denoted by circles and phosphorylation of the α-subunit by triangles. Open symbols show results obtained using cyclic GMP-dependent protein kinase and closed symbols results using cyclic AMP-dependent protein kinase. Activity measurements were carried out at pH 6.8. Activation was only 1.6-fold at pH 8.2, when it was 10-fold at pH 6.8.

Cyclic AMP-dependent protein kinase has been shown to phosphorylate one serine residue on the α-subunit and one serine residue on the β-subunit of phosphorylase kinase [23,24]. The tryptic phosphopeptide from the α-subunit (40 amino acids) is much larger than that from the β-subunit (9 amino acids) and the two sites are therefore easily distinguished by gel filtration [23]. The tryptic phosphopeptides

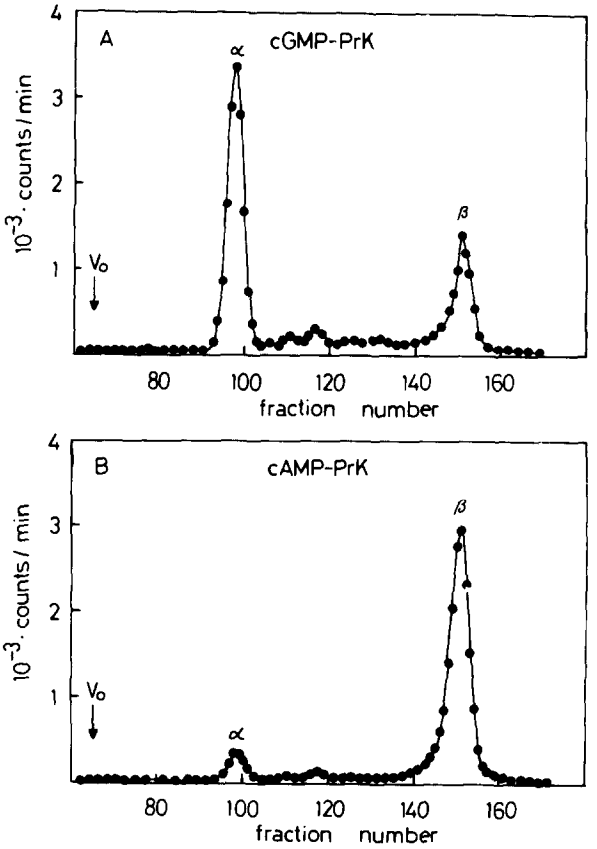

Fig.4. Gel filtration of tryptic phosphopeptides on Sephadex G-50 Superfine (150 × 1.4 cm). Phosphorylase kinase (0.4 mg) was phosphorylated by (A) cyclic GMP-dependent protein kinase (cGMP-PrK), or (B) cyclic AMP-dependent protein kinase (cAMP-PrK). The sample phosphorylated by cGMP-PrK contained 0.83 molecules of phosphate in the α- and 0.33 in the β-subunit. The sample phosphorylated by cAMP-PrK contained 0.11 molecules of phosphate in the α-subunit and 0.50 in the β-subunit. Incubations were terminated with 5% trichloroacetic acid and the precipitated protein washed four times with water to remove trichloroacetic acid and [γ-^{32}P]ATP. The precipitate was resuspended in 0.1 ml of ammonium bicarbonate (0.1 M), incubated with 0.01 ml of trypsin (0.1 mg/ml) for 24 h at 37°C, and subjected to gel filtration. The flow rate was 10 ml/h and 1.0 ml fractions were collected.

obtained after phosphorylation by cyclic GMP or cyclic AMP-dependent protein kinase were found to be identical (fig.4).

3.2. *Dephosphorylation and inactivation of phosphorylase kinase by protein phosphatase-1 and protein phosphatase-2*

The *initial* rate of dephosphorylation of the β-subunit by protein phosphatase-1 was 50-fold faster than the dephosphorylation of the α-subunit, whereas the *initial* rate of dephosphorylation of the α-subunit by protein phosphatase-2 was 10–20-fold faster than the β-subunit as expected [25,26]. The inactivation of phosphorylase kinase showed an excellent correlation with the loss of phosphate from the β-subunit (fig.5). After a 15 min incubation with protein phosphatase-1 the activity had almost returned to the level of the dephosphorylated enzyme and the β-subunit was largely dephosphorylated, but the α-subunit was still 80–90% phosphorylated. Conversely, after a 15 min incubation with protein phosphatase-2 the activity and phosphate in the β-subunit had decreased by 20–30%, but the α-subunit was 80% dephosphorylated.

4. Discussion

Incubation of phosphorylase kinase with cyclic AMP-dependent protein kinase at low concentrations of Mg^{2+} (1–2 mM) leads to a rapid phosphorylation of one serine on the β-subunit, and a slower phosphorylation of one serine on the α-subunit [12,23,24]. We reported previously that most, if not all, of the activation observed at physiological pH (6.8) was associated with the phosphorylation of the β-subunit [12], a result confirmed by Singh and Wang [27]. However, Hayakawa et al. [28] reported that the activity of phosphorylase kinase was still increasing at pH 6.8, after the phosphorylation of the β-subunit was complete, and they suggested the α-subunit also played a part in the activation process [28]. The discovery that cyclic GMP-dependent protein kinase phosphorylates the same two serine residues, but at quite different relative rates, and the availability of two phosphorylase kinase phosphatases which are relatively specific for the dephosphorylation of the two sites, has resolved this question. At pH 6.8 in the presence of saturating concentrations of Ca^{2+} there is a very strong correlation between the activity of the enzyme and the state of phosphorylation of the β-subunit. The site on the α-subunit that is phosphorylated *preferentially* by either cyclic AMP- or cyclic GMP-dependent protein kinase does not influence the activity under these conditions.

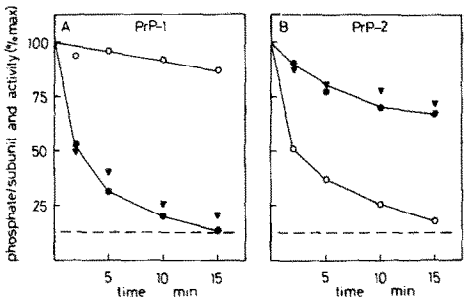

Fig.5. Dephosphorylation and inactivation of phosphorylase kinase by (A) protein phosphatase-1 (PrP-1) and (B) protein phosphatase-2 (PrP-2). [^{32}P]Phosphorylase kinase containing 0.84 molecules of phosphate in the α-subunit and 0.85 molecules in the β-subunit was prepared by phosphorylation with cyclic AMP-dependent protein kinase, and incubated with protein phosphatase-1 or protein phosphatase-2 as described under Materials and methods. The open and closed circles show the % phosphate remaining the α and β-subunits respectively. The closed triangles show the activity at pH 6.8, and the broken line denotes the activity of the dephosphorylated form of the enzyme.

Incubation of phosphorylase kinase with cyclic AMP-dependent protein kinase at high concentrations of Mg^{2+} (10 mM) for prolonged periods leads to the incorporation of 7–9 molecules of phosphate per αβγδ unit and activates the enzyme to a level 2–3-fold higher than is obtained after phosphorylation to 2 molecules per αβγδ unit at low Mg^{2+} [27]. Nearly all the additional phosphate is incorporated into the α-subunit. It therefore seems clear that the phosphorylation of a site(s) on the α-subunit *distinct* from that phosphorylated preferentially by cyclic AMP-dependent protein kinase, contributes to the activation of phosphorylase kinase under these conditions.

The two sites on the α and β-subunits phosphorylated by cyclic AMP-dependent protein kinase at low Mg^{2+} are partially phosphorylated in vivo in response to adrenalin [29], and the total phosphate bound covalently to phosphorylase kinase increases by the amount expected from the extent of phosphorylation of these sites (about one molecule per αβγδ unit [30]). The physiological significance of the large number of phosphates that can be introduced into the α-subunit by incubation with cyclic AMP-dependent protein kinase at high Mg^{2+} in vitro is therefore uncertain.

The *initial* rate of phosphorylation of phosphorylase kinase by cyclic GMP-dependent protein kinase is about 40–50-fold slower than that catalysed by cyclic AMP-dependent protein kinase, but the rate of activation is 150-fold slower. This large difference presumably explains the original failure to detect activation of phosphorylase kinase by cyclic GMP-dependent protein kinase [2–4]. Since the levels of cyclic GMP-dependent protein kinase in skeletal muscle are extremely low [31], it is most unlikely that the phosphorylation of phosphorylase kinase by this enzyme occurs in vivo. On the other hand, the current information about the specificity of cyclic GMP-dependent protein kinase suggests that this enzyme may be of general use in elucidating the functions of particular phosphorylation sites in enzymes that are phosphorylated at multiple positions.

Phosphorylase kinase is now the third protein where cyclic AMP and cyclic GMP-dependent protein kinase have been shown to phosphorylate the same two sites but at quite different relative rates. Histone H2B is phosphorylated by both enzymes at serine-32 and serine-36 [32]. Serine-36 is phosphorylated 6-fold more rapidly than serine-32 by cyclic AMP-dependent protein kinase [24], whereas serine-32 is phosphorylated 10-fold more rapidly than serine-36 by cyclic GMP-dependent protein kinase [7,33]. G-substrate, a protein present in the cerebellum, is one of the few proteins phosphorylated more rapidly by cyclic GMP-dependent protein kinase than by cyclic AMP-dependent protein kinase [34,35]. This protein is phosphorylated on two threonine residues by both protein kinases, termed site-1 and site-2. Site-2 is phosphorylated slightly faster than site-1 by cyclic GMP-dependent protein kinase but site-1 is phosphorylated 4-fold faster than site-2 by cyclic AMP-dependent protein kinase [36]. The specificity determinants of cyclic AMP and cyclic GMP-dependent protein kinase therefore appear to be similar but not identical [37], and their combined use is likely to be important in the analysis of other proteins that are multiply phosphorylated.

Acknowledgements

This work was supported by a Programme grant from the Medical Research Council, London and by the British Diabetic Association.

References

[1] Kuo, J. F., Shoji, M. and Kuo, W. N. (1978) Ann. Rev. Pharmacol. Toxicol. 18, 341–355.
[2] Inoue, M., Kishimoto, A., Takai, Y. and Nishizuka, Y. (1976) J. Biol. Chem. 251, 4476–4478.
[3] Takai, Y., Nishiyama, K., Yamamura, H. and Nishizuka, Y. (1975) J. Biol. Chem. 250, 4690–4695.
[4] Kuo, J. F., Kuo, W., Shoji, M., Davis, C. W., Seery, V. L. and Donnelly, T. E. (1976) J. Biol. Chem. 251, 1759–1766.
[5] Lincoln, T. M. and Corbin, J. D. (1977) Proc. Natl. Acad. Sci. USA 74, 3239–3243.
[6] Khoo, J. C., Speery, P. J., Gill, G. N. and Steinberg, D. (1977) Proc. Natl. Acad. Sci. USA 74, 4843–4847.
[7] Hashimoto, E., Takeda, M., Nishizuka, Y., Hamana, K. and Iwai, K. (1976) J. Biol. Chem. 251, 6287–6293.
[8] Blumenthal, D. K., Stull, J. T. and Gill, G. N. (1978) J. Biol. Chem. 253, 334–336.
[9] Lincoln, T. M. and Corbin, J. D. (1978) J. Biol. Chem. 253, 337–339.
[10] Hardie, D. G. and Guy, P. S. (1980) Eur. J. Biochem., in the press.
[11] Guy, P. S., Cohen, P. and Hardie, D. G. (1980) Eur. J. Biochem., submitted.
[12] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[13] Fischer, E. H. and Krebs, E. G. (1958) J. Biol. Chem. 231, 68–71.
[14] Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1974) Methods Enzymol. 38, 299–308.
[15] Nimmo, G. A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[16] Stewart, A. A., Goris, J., Hemmings, B. A. and Cohen, P. (1980) Eur. J. Biochem., submitted.
[17] Nimmo, G. A. and Cohen, P. (1978) Eur. J. Biochem. 87, 353–365.
[18] Foulkes, J. G. and Cohen, P. (1980) Eur. J. Biochem. 105, 195–203.
[19] Walter, U., Miller, P., Wilson, F., Meakes, D. and Greengard, P. (1980) J. Biol. Chem. 255, 3757–3762.
[20] Shenolikar, S., Cohen, P. T. W., Cohen, P., Nairn, A. C. and Perry, S. V. (1979) Eur. J. Biochem. 100, 329–337.
[21] Cohen, P. (1980) Eur. J. Biochem., in the press.
[22] Cohen, P., Nimmo, G. A. and Antoniw, J. F. (1977) Biochem. J. 162, 435–444.
[23] Cohen, P., Watson, D. C. and Dixon, G. H. (1975) Eur. J. Biochem. 51, 79–92.
[24] Yeaman, S. J., Cohen, P., Watson, D. C. and Dixon, G. H. (1977) Biochem. J. 162, 411–421.
[25] Antoniw, J. F. and Cohen, P. (1976) Eur. J. Biochem. 68, 45–54.
[26] Cohen, P. (1978) Curr. Top. Cell. Regul. 14, 117–196.
[27] Singh, T. J. and Wang, J. A. (1977) J. Biol. Chem. 252, 625–632.
[28] Hayakawa, T., Perkins, J. P. and Krebs, E. G. (1973) Biochemistry 12, 574–580.
[29] Yeaman, S. J. and Cohen, P. (1975) Eur. J. Biochem. 51, 93–104.

[30] Nimmo, H. G. and Cohen, P. (1977) Adv. Cyclic Nucl. Res. 8, 145–266.
[31] Lincoln, T. M., Hall, C. L., Park, C. R. and Corbin, J. D. (1976) Proc. Natl. Acad. Sci. USA 73, 2559–2663.
[32] Kuroda, Y., Hashimoto, E. and Nishizuka, Y. (1976) Biochem. Biophys. Res. Commun. 71, 629–635.
[33] Glass, D. B. and Krebs, E. G. (1979) J. Biol. Chem. 254, 9728–9738.
[34] Schlichter, D. J., Casnellie, J. E. and Greengard, P. (1978) Nature 273, 61–62.
[35] Aswad, D. and Greengard, P. (1980) J. Biol. Chem., submitted.
[36] Aitken, A., Bilham, T., Cohen, P., Aswad, D. and Greengard, P. (1980) J. Biol. Chem., submitted.
[37] Glass, D. B. and Krebs, E. G. (1980) Ann. Rev. Pharmacol. Toxicol. 20, 363–388.