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MULTIPLE FORMS OF PHOSPHOPROTEIN PHOSPHATASE FROM RABBIT RETICULOCYTES

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Summary

Three major phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) activities (I—III) from reticulocytes were resolved by DEAE-cellulose chromatography and fractionated further by chromatography on Sephacryl S-200. The major activity in each fraction had similar properties and was designated the B form (M_r 270 000). This form constituted approximately 80% of the total phosphatase activity; approximately 15% of the activity was present as the C form (M_r 140 000). Two other forms, D (M_r 180 000) and A ($M_r > 500\,000$) were observed in minor amounts. All of the activities dephosphorylated histone phosphorylated by cyclic AMP-regulated kinases and casein phosphorylated by casein kinase II. When the B and C forms were examined further, a broad pH optimum between 7 and 8 was observed with histone; manganese stimulated dephosphorylation of histone with the B form but was required with the C form. Using casein as substrate, two pH optima, 5.6 and 7.5, were observed with the B form in the presence of manganese. In the absence of manganese, phosphatase activity with casein was observed only at pH 5.6. The C form was minimally active with casein. Phosphatase B was stimulated by treatment with trypsin and by freezing and thawing in the presence of mercaptoethanol; phosphatase C was either not affected or inhibited by these treatments. At high concentrations (4 mM) several small molecular weight compounds including ATP, GTP, glucose 6-phosphate and NaF were found to inhibit the B and the C forms. Cyclic AMP and hemin had little or no effect on the activities of phosphatase B and C with casein or histone as substrate.

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

Introduction

Phosphoprotein phosphatases (phosphoprotein phosphohydrolases, EC 3.1.3.16) have been shown to exist in multiple forms in a number of tissues including rabbit liver [1—4], rabbit skeletal muscle [5,6], rat liver [7,8], rat cerebral cortex [9] and canine heart [10]. These enzymes dephosphorylate phosphorylase which has been phosphorylated by phosphorylase kinase [1—4, 6,7] and histone and casein phosphorylated by the cyclic AMP-regulated protein kinases [2—6,8—10] as well as a number of other phosphoprotein substrates. The phosphatase activities from liver could be activated by ethanol precipitation [1], trypsin treatment [11] and freeze-thaw in the presence of mercaptoethanol [3]. Activation by ethanol precipitation or freeze-thaw resulted in the concomitant conversion of the activities to a lower molecular weight form. A similar activation has been observed in rabbit skeletal muscle [12] and canine heart [13]. The activation of phosphoprotein phosphatase appeared to be mediated by the release of an inhibitory protein from the less active, higher molecular weight complex. Support for this concept was obtained by the identification of two heat-stable inhibitors of phosphoprotein phosphatase [1,14—20]. The inhibitor proteins have been identified in several tissues and phosphorylation of one of the proteins by the cyclic AMP-regulated protein kinase was shown to increase the inhibitory activity [15,18,20].

Rabbit reticulocytes contain both cyclic AMP-regulated and cyclic nucleotide-independent protein kinases. Two forms of the cyclic AMP-regulated protein kinase (types I and II) were resolved from the postribosomal supernatant fraction by chromatography on DEAE-cellulose [21,22]. Two cyclic nucleotide-independent protein kinases, casein kinase I and casein kinase II, have been purified and partially characterized [22,23]. The recognition sequences for the two cyclic nucleotide-independent protein kinases in α_{s1} and β casein were shown to be unique and different from that of the cyclic AMP-regulated protein kinases [24,25].

In this report we describe the isolation and characterization of the phosphoprotein phosphatases from rabbit reticulocytes which dephosphorylase histone phosphorylated by cyclic AMP-regulated protein kinases and casein phosphorylated by casein kinase II.

Experimental procedures

Materials. [γ-^{32}P]ATP was prepared as described by Hathaway et al. [22]. The specific activity of the ATP used in the assays ranged from 70 to 680 Ci/mol. Casein (Matheson, Coleman and Bell) was prepared as described by Reimann et al. [26]. Histone IIA, trypsin and soybean trypsin inhibitor were purchased from Sigma.

Preparation of protein kinases. Protein kinases were prepared from the postribosomal supernatant fraction of rabbit reticulocytes by chromatography on DEAE-cellulose and phosphocellulose as described by Hathaway et al. [22]. The cyclic AMP-regulated protein kinase (type II) was used to phosphorylate histone IIA and casein kinase II was used to phosphorylate casein. One unit of protein kinase incorporated 1 pmol of phosphate/min at 30°C.

Phosphorylation of histone and casein and assay for phosphoprotein phosphatase. Histone was phosphorylated by the type II cyclic AMP-dependent protein kinase and casein kinase II with [γ-^{32}P]ATP as described in detail elsewhere [27]. Phosphoprotein phosphatase activities were measured by release of radioactive phosphate using the method of Parvin and Smith [28] as modified by Kato and Bishop [29] and described previously [27]. Dephosphorylation of histone and casein was conducted at pH 7.0; dephosphorylation of casein was also examined at pH 5.6.

Purification of phosphoprotein phosphatase activities. Phosphoprotein phosphatase activities were purified from the postribosomal supernate from rabbit reticulocytes by DEAE-cellulose chromatography and gel filtration on Sephacryl S-200 (Pharmacia) using phosphohistone and phosphocasein as substrate [27]. Three peaks of phosphatase activity were observed after DEAE-cellulose chromatography. Fraction I eluted from 0.05 M to 0.10 M NaCl, fraction II from 0.10 M to 0.20 M and fraction III from 0.25 M to 0.30 M NaCl. These fractions were individually concentrated and applied to a Sephacryl S-200 column. The ensuing activities were described according to the elution profile as A ($M_r > 500\,000$), B (M_r 270 000), C (M_r 140 000) and D (M_r 180 000).

Treatment of phosphatase activities with trypsin. The effects of limited proteolysis on the phosphatase activities from the S-200 step were examined by incubation of the enzymes (0.15 ml) with 2 μg/ml of trypsin (diphenyl carbamyl chloride treated) in the presence of 5 mg/ml bovine serum albumin in a final volume of 0.2 ml. After incubation for 20 min at 30°C, a 10-fold excess of soybean trypsin inhibitor was added to inhibit proteolytic digestion. 0.02 ml aliquots were removed and assayed for phosphatase activity as described previously.

Freeze-thaw treatment in the presence of mercaptoethanol. The effects of freezing and thawing on the phosphatase activities from the S-200 chromatography step were examined by adding 0.005 ml of 0.8 M 2-mercaptoethanol to 0.015 ml aliquots of the pooled enzyme fractions. The samples were placed at —20°C for 20 min, thawed and assayed for phosphatase activity.

Results

Resolution of phosphoprotein phosphatases from reticulocytes

Phosphoprotein phosphatase activities from rabbit reticulocytes were resolved into three major fractions (I—III) by chromatography on DEAE-cellulose. The fractions were individually pooled and fractionated further by gel filtration on Sephacryl S-200. The phosphatase activities were identified by molecular weight as form A (500 000), B (270 000), C (140 000) and D (180 000). The A and B forms were observed in all three fractions, the C form was in fractions I and II and the D form in fraction III only [27]. The B form comprised approximately 80% of the total phosphatase activity and the C form approximately 15%. Both the A and D forms were minor activities.

Effects of pH on phosphatase activities

The phosphatase fractions were assayed with histone and casein at pH values

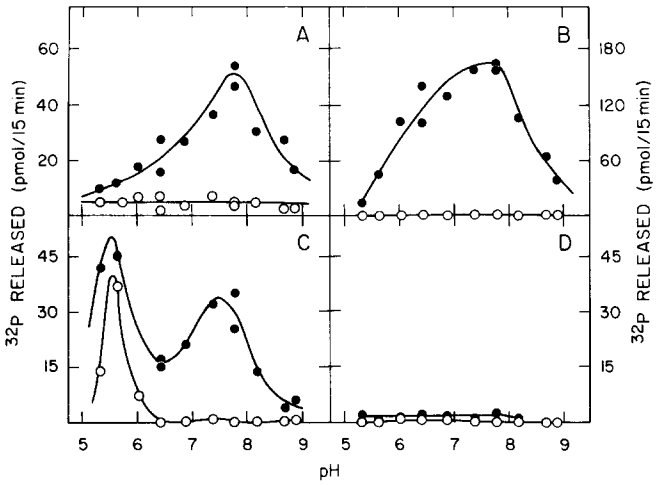

Fig. 1. Effects of pH on phosphatase activity. The phosphatase activity of IIB and IIC was determined as a function of pH. Assay mixtures contained 50 mM buffer, 20 mM 2-mercaptoethanol and 1000 pmol of [^{32}P]histone or casein. Incubation time was 15 min. The buffers used were: pH 5.0—6.5, Mes; pH 6.5—7.5, Mops; pH 7.5—9.0, Tris-HCl. Plus 5 mM $MnCl_2$ (●———●); $MnCl_2$ omitted (○———○). (A) IIB assayed with histone; (B) IIC assayed with histone; (C) IIB assayed with casein; (D) IIC assayed with casein.

ranging from 5.4 to 9. Results obtained with IIB and IIC are shown in Fig. 1. When histone was used as substrate both fractions had optimal activity between pH 7.0 and 8.0 when assayed with Mn^{2+}. When fraction IIB was assayed with casein in the presence of Mn^{2+}, two pH optima were observed, 5.6 and 7.5. In contrast, a single optimum was observed at pH 5.6 when Mn^{2+} was omitted from the assay. Fraction IIC had minimal activity with casein in the presence or absence of Mn^{2+}. Thus the phosphatase activities are dependent on substrate, Mn^{2+} and pH which vary with the enzymic activity examined.

Substrate specificity

As shown in Table I, all of the phosphatase activities dephosphorylated both

TABLE I

SUBSTRATE SPECIFICITY OF PHOSPHATASE ACTIVITIES

An aliquot (0.015 ml) of the peak fraction from each of the indicated phosphatase fractions was assayed with histone and casein at pH 7.0 and casein at pH 5.6 in the presence and absence of 5 mM $MnCl_2$.

Fraction		Phosphatase activity (units/ml)					
		Histone		Casein, pH 7.0		Casein, pH 5.6	
		$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$	$+Mn^{2+}$	$-Mn^{2+}$
I	A	8	5	0	8	167	158
	B	27	15	44	36	1570	1532
	C	55	4	231	0	174	12
II	A	153	37	153	0	160	96
	B	385	135	351	16	390	551
	C	328	0	54	0	8	6
III	A	126	21	47	0	0	30
	B	261	99	304	0	126	239
	D	255	43	541	3	215	123

histone and casein. With these substrates phosphatase IB was stimulated up to 2-fold by 5 mM Mn^{2+} when assayed at pH 7.0. The activity of IB with casein was 20—40-fold higher when assayed at pH 5.6 than when assayed at pH 7.0. Manganese had no effect on dephosphorylation of casein at the lower pH. IC was completely dependent on Mn^{2+} for activity with either histone or casein. In the presence of 5 mM Mn^{2+}, dephosphorylation of casein was similar at pH 5.6 and 7.0, and 5-fold higher than that of histone. The activities designated IB and IC appeared to be unique by the following two criteria: Mn^{2+} had little effect on IB whereas IC required Mn^{2+}; the activity of IB with casein was 20—40-fold higher when assayed at pH 5.6 than at 7.0, while the activity of IC was minor at both pH 5.6 and 7.0.

IIB dephosphorylated both histone and casein. Phosphatase activity with histone was stimulated 1—2-fold by the addition of 5 mM $MnCl_2$ to the reaction mixtures. Dephosphorylation of casein at pH 7.0 was dependent on Mn^{2+}, whereas at pH 5.6 the cation had little effect or was slightly inhibitory (Table I). IIC had an absolute requirement for Mn^{2+} when assayed at pH 7.0 with histone or casein as substrate. At pH 5.6, little if any enzymic activity was observed with casein either in the presence or absence of Mn^{2+}.

IIIB had properties very similar to those of IIB and both enzymic activities were similar in the dephosphorylation of histone and casein. Mn^{2+} stimulated the activities with histone 2—3-fold; however, the cation was required for the dephosphorylation of casein at pH 7.0. Phosphatase activity with casein was decreased at pH 5.6 and Mn^{2+} appeared to inhibit the activity. IIID dephosphorylated histone in the absence of manganese and the activity was stimulated 4—5-fold by the addition of 5 $MnCl_2$. Mn^{2+} was required for the dephosphorylation of casein at pH 7.0. In the presence of Mn^{2+}, the dephosphorylation of casein was greater than that observed with histone. When assayed at pH 5.6, the rate of dephosphorylation with casein was lower than that observed at pH 7.0 and Mn^{2+} had an inhibitory effect on the activity of IIID at the lower pH.

Effects of trypsin on phosphatase activity

To examine the possible role of proteolysis in the activation of the phosphoprotein phosphatases from reticulocytes, IB and IC were examined before and after limited treatment with trypsin. IB was activated at least 6-fold by trypsin when assayed with casein at pH 7.0 (Table II); however, trypsin had no effect on the activity when assayed with histone or with casein at pH 5.6. Limited proteolysis of IC had no effect on the phosphatase activity or resulted in inhibition.

Effects of freezing and thawing

Phosphoprotein phosphatases from liver and skeletal muscle have been shown to be activated by freezing and thawing in the presence of mercaptoethanol [3,5]. When IB and IC were frozen and thawed in the presence of 0.2 M mercaptoethanol, a 3—6-fold stimulation of IB was observed when assayed with histone and casein at pH 7.0 (Table III). No increase in activity was seen when IB was examined with casein at pH 5.6; however, the activity at this pH was already 6-fold greater than that at pH 7.0. Thus the activity of the freeze-thaw-activated enzyme with casein at pH 7.0, was equivalent to non-

TABLE II

EFFECTS OF LIMITED PROTEOLYSIS ON PHOSPHATASE ACTIVITY

IB and IC were preincubated in the presence (+T) and absence (—T) of trypsin. After addition of soybean trypsin inhibitor, the phosphatase activities were assayed with histone and casein at pH 7.0 and casein at pH 5.6. Incubation mixtures contained 0.9 unit of IB or 1.0 unit of IC.

Substrate	$MnCl_2$	Phosphatase activity (units/ml)			
		IB		IC	
		—T	+T	—T	+T
Histone (pH 7.0)	—	29	36	6	4
	+	44	54	52	44
Casein (pH 7.0)	—	39	247	0	0
	+	15	221	44	41
Casein (pH 5.6)	—	1189	1195	72	7
	+	1156	1277	84	22

treated enzyme at pH 5.6. Freeze-thaw inhibited the activity of IC under all assay conditions.

Analysis of effector compounds on phosphatase activity

Various compounds known to affect cell metabolism were examined with the partially purified phosphatases IB, IC, IIB and IIIB. 0.025 mM hemin and 4 mM cyclic AMP had little effect on dephosphorylation of histone or casein at pH 7.0 (Table IV). Hemin was slightly inhibitory to most of the phosphatase fractions. High concentrations of cyclic AMP caused a slight stimulation (38%) of phosphatase activities IIB and IIIB when histone was used as substrate, no effect was observed when casein was used as substrate.

Addition of glucose 6-phosphate, ATP and GTP (4 mM) resulted in significant inhibition (64—100%) of IB, IIB and IIIB when casein was used as substrate. The phosphatase. activity of these same fractions with histone was

TABLE III

EFFECTS OF FREEZE-THAW ON PHOSPHATASE ACTIVITY

Phosphatase activities IB and IC were frozen and thawed in the presence of 0.2 M mercaptoethanol. After freeze-thaw treatment the fractions were assayed for phosphatase activity. Control samples which remained at 4°C were also assayed. Incubations contained 0.1 unit of IB or 0.2 unit of IC.

Substrate	$MnCl_2$	Phosphatase activity (units/ml)			
		IB		IC	
		4°C	—20°C	4°C	—20°C
Histone (pH 7.0)	—	6	36	3	1
	+	8	26	10	1
Casein (pH 7.0)	—	58	335	14	4
	+	29	101	110	7
Casein (pH 5.6)	—	293	320	12	15
	+	297	220	52	13

TABLE IV

ANALYSIS OF EFFECTOR COMPOUNDS ON THE DEPHOSPHORYLATION OF HISTONE AND CASEIN

Phosphatases resolved by chromatography on S-200 were assayed in the presence of various effector compounds. Phosphatase activity was determined with histone and casein at pH 7.0 in the presence of 5 mM $MnCl_2$, except when indicated. Sufficient phosphatase activity was added to release 5—15% of the protein-bound phosphate during a 15 min incubation. Final concentrations of the compounds are indicated. Activity is expressed relative to the control containing no additions.

Addition	Concentration (mM)	Relative activity							
		IB		IC		IIB		IIIB	
		Histone	Casein (pH 7.0)	Histone	Casein (pH 7.0)	Histone	Casein (pH 7.0)	Histone	Casein (pH 7.0)
None		100	100	100	100	100	100	100	100
Hemin	0.025	83	73	62	75	89	111	90	88
Glc-6-*P*	4.0	52	9	17	58	51	14	52	34
ATP	4.0	64	36	11	88	47	2	47	27
GTP	4.0	56	9	6	113	40	0	40	19
Cyclic AMP	4.0	84	114	66	108	137	110	138	95
NaF	4.0	56	45	38	96	27	35	28	48
$MnCl_2$ omitted		38	54	3	0	11	0	11	0

inhibited to a lesser extent (40—50%) by the same compounds. In contrast, the activity of IC with histone was inhibited to a greater extent (83—94%) than the activity with casein (12—42%). In all three cases, the greatest inhibition was observed with GTP, and ATP was more inhibitory than glucose-6-phosphate. 4 mM NaF was moderately inhibitory with all of the phosphatase activities.

Discussion

The substrate specificity of individual phosphoprotein phosphatase activities was monitored using histone and casein as substrate. All of the phosphoprotein phosphatase activities dephosphorylated both of the substrates. In general, Mn^{2+} stimulated the dephosphorylation of histone and was an absolute requirement for the dephosphorylation of casein at pH 7.0. At pH 5.6, Mn^{2+} had little effect on the dephosphorylation of casein.

Phosphatase IB was stimulated by treatment with trypsin and by freezing and thawing in the presence of mercaptoethanol. In contrast, the C form was not stimulated by either of these treatments and in some cases inhibition was observed. IB was also stimulated when assayed with casein at pH 5.6. Since phosphatase activity of IB at pH 5.6 was not increased by trypsin or freeze-thaw treatment, the mechanism of activation at the lower pH may be similar to that observed with the trypsin and freeze-thaw treatments. Additional data suggested that IC was an activated form of IB. In preliminary studies we have observed conversion of the B form to the C form by an endogenous calcium-activated protease; a 5-fold increase in phosphatase activity accompanied this conversion (unpublished data).

Others have shown that the high molecular weight forms of phosphorylase phosphatase and histone phosphatase were activated by treatment with trypsin and urea, by precipitation with room temperature ethanol and by partial proteolysis during homogenization of the liver [3,5,11,13,30,31]. A concomitant reduction in molecular weight was observed also.

It is interesting to note that the major form of phosphatase activity dephosphorylated histone phosphorylated by the cyclic AMP-regulated protein kinase and casein phosphorylated by casein kinase II. It has been shown previously that the sites modified by the cyclic AMP-regulated protein kinases were different from the sites phosphorylated by casein kinase II [24]. Recognition determinants for the cyclic AMP-regulated protein kinases have been identified as multiple basic residues, in particular arginine, on the NH_2-terminal side of the target serine residue [32]. The recognition sequence for casein kinase II in α_{s1} and β-casein has been identified as Thr-Glu-Asp [25]. Thus the amino acid sequence is important in determination of the site of phosphorylation for the protein kinases, whereas the phosphatases appear to be more relaxed with regard to primary structure. An alternative explanation would be that each of the phosphatase activities contains more than one enzymic activity. This appears unlikely since highly purified preparations of the phosphatases prepared by further chromatography on histone-Sepharose have been shown to contain both the histone and casein activities (unpublished data).

At high concentrations (4 mM), glucose 6-phosphate, ATP and GTP were inhibitory with phosphatase IB, IIB, IIIB and IC. Inhibition was greater when casein was used as substrate. Kato et al. [33] described the inactivation of phosphatase from rabbit skeletal muscle by ATP. The activity could be restored by subsequent incubation with Mn^{2+}, Mg^{2+} or Ca^{2+}, suggesting that ATP was chelating a required divalent metal ion resulting in a loss of activity. Similar inactivation and reactivation has been observed with phosphoprotein phosphatase activities from canine heart [31,34]. Cyclic AMP and hemin had very little effect on the B and C forms of the phosphatase, although in other studies hemin has been shown to stimulate a minor component, phosphatase IA [35]. 4 mM NaF inhibited most of the activities to a moderate extent.

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References

1 Brandt, H., Lee, E.Y.C. and Killilea, S.D. (1975) Biochem. Biophys. Res. Commun. 63, 950—956
2 Khandelwal, R.L., Vandenheede, J.R. and Krebs, E.G. (1976) J. Biol. Chem. 251, 4850—4858
3 Kobayashi, M., Kato, K. and Sato, S. (1975) Biochim. Biophys. Acta 377, 343—355
4 Meisler, M.H. and Langan, T.A. (1969) J. Biol. Chem. 244, 4961—4968
5 Kobayashi, M. and Kato, K. (1977) J. Biochem. 81, 93—97
6 Antoniw, J.F., Nimmo, H.G., Yeaman, S.J. and Cohen, P. (1977) Biochem. J. 162, 423—433
7 Abe, N. and Tsuiki, S. (1974) Biochim. Biophys. Acta 350, 383—391
8 Ray, K.P. and England, P.J. (1976) Biochem. J. 157, 369—380
9 Meano, H. and Greengard, P. (1972) J. Biol. Chem. 247, 3269—3277
10 Li, H.-C. (1975) FEBS Lett. 55, 134—137

11 Lee, E.Y.C., Brandt, H., Cupolang, Z.L. and Killilea, S.D. (1976) Adv. Enzyme Regul. 14, 467—490
12 Kato, K., Kobayashi, M. and Sato, S. (1974) Biochim. Biophys. Acta 371, 89—101
13 Hsaio, K-J., Chan, W.W.S. and Li, H.-C. (1977) Biochim. Biophys. Acta 483, 337—347
14 Brandt, H., Killilea, S.D. and Lee, E.Y.C. (1974) Biochem. Biophys. Res. Commun. 61, 598—604
15 Huang, F.L. and Glinsman, W.H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3004—3008
16 Huang, F.L. and Glinsman, W.H. (1976) FEBS Lett. 62, 326—329
17 Huang, F.L. and Glinsman, W.H. (1976) Eur. J. Biochem. 70, 419—425
18 Huang, F.L., Tao, S. and Glinsman, W.H. (1977) Biochem. Biophys. Res. Commun. 78, 615—623
19 Cohen, P., Nimmo, G.A. and Antoniw, J.F. (1977) Biochem. J. 162, 435—444
20 Nimmo, G.A. and Cohen, P. (1978) Eur. J. Biochem. 87, 353—365
21 Traugh, J.A. and Traut, R.R. (1974) J. Biol. Chem. 249, 1207—1212
22 Hathaway, G.M., Lundak, T.S., Tahara, S.M. and Traugh, J.A. (1979) Methods Enzymol. 60, 495—511
23 Hathaway, G.M. and Traugh, J.A. (1979) J. Biol. Chem. 254, 762—768
24 Tuazon, P.T. and Traugh, J.A. (1978) J. Biol. Chem. 253, 1746—1748
25 Tuazon, P.T., Bingham, E.W. and Traugh, J.A. (1979) Eur. J. Biochem. 94, 497—504
26 Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1971) J. Biol. Chem. 246, 1986—1995
27 Mumby, M. and Traugh, J.A. (1979) Methods Enzymol. 60, 522—534
28 Parvin, R. and Smith, R.A. (1969) Anal. Biochem. 27, 65—72
29 Kato, K. and Bishop, J.S. (1972) J. Biol. Chem. 247, 7420—7429
30 Lee, E.Y.C., Mellgren, R.L., Killelea, S.D. and Aylward, J.H. (1978) Proceedings of 11th FEBS Meeting, Vol. 42, 327—346
31 Li, H.-C. and Hsiao, K.-J. (1977) Eur. J. Biochem. 77, 383—391
32 Kemp, B.E., Graves, D.J., Benjamini, E. and Krebs, E.G. (1977) J. Biol. Chem. 252, 4888—4894
33 Kato, K., Kobayashi, M. and Sato, S. (1975) J. Biochem. 72, 811—815
34 Li, H.-L., Hsiao, K.-J. and Chan, W.W.S. (1978) Eur. J. Biochem. 84, 215—225
35 Mumby, M. and Traugh, J.A. (1977) Fed. Proc. Abstr. 36, 778