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The inhibitor protein of the cyclic AMP-dependent protein kinase–catalytic subunit interaction

Composition of multiple complexes

Scott M. VAN PATTEN,* Agnes HOTZ,† Volker KINZEL† and Donal A. WALSH*‡

*Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616, U.S.A., and †German Cancer Research Center, Institute of Experimental Pathology, D-6900 Heidelberg, Federal Republic of Germany

It has been previously demonstrated that the combination of pure preparations of the inhibitor protein of the cyclic AMP-dependent protein kinase and the catalytic subunit of this enzyme resulted in the formation of multiple complexes [Van Patten, Fletcher & Walsh (1986) J. Biol. Chem. **261**, 5514–5523]. In the present study it is demonstrated that these multiple species occur because the bovine heart protein kinase preparation contains multiple forms of catalytic subunit [Kinzel, Hotz, König, Gagelmann, Pyerin, Reed, Köbler, Hofmann, Obst, Gensheimer, Goldblatt & Shaltiel (1987) Arch. Biochem. Biophys. **253**, 341–349].

INTRODUCTION

The inhibitor protein of the cyclic AMP-dependent protein kinase acts by direct interaction at the catalytic site with the formation of an inhibitor protein–catalytic subunit complex (Ashby & Walsh, 1972). The formation of this complex is ATP-stimulated (Whitehouse & Walsh, 1983*a*), and a significant component of the affinity of the inhibitor protein for the kinase is that it possesses several of the same recognition signals that dictate protein substrate binding and specificity (Scott *et al.*, 1985, 1986; Cheng *et al.*, 1985, 1986; Reed *et al.*, 1987). In a previous study, a non-denaturing gel-electrophoresis system was established that permitted the identification of complex-formation (Van Patten *et al.*, 1986). This led to the discovery that with pure constituents of rabbit skeletal-muscle inhibitor protein and bovine heart protein kinase catalytic subunit at least two major and one minor species of complex resulted. All complexes contained a 1:1 molar stoichiometry of both proteins. Although it is known that the inhibitor protein can exist in two forms, designated I and I′ (McPherson *et al.*, 1979), it was demonstrated (Van Patten *et al.*, 1986) that all complexes only contained the physiological I form. These data suggested the possibility that the heterogeneity of inhibitor protein–catalytic subunit complex-formation could have arisen as a consequence of multiple species of catalytic subunit. Multiple forms of catalytic subunit have been proposed from earlier studies (Bechtel *et al.*, 1977; Chen & Walsh, 1971; Reed *et al.*, 1983), but more recently have been separated and characterized (Kinzel *et al.*, 1987). By use of the chromatographic procedure of the latter study, it is now demonstrated that the multiple complexes of inhibitor protein–catalytic subunit, as observed by non-denaturing gel electrophoresis, are indeed a consequence of multiple species of catalytic subunit in the purified protein kinase preparation. The characteristics of the two major complexes are presented.

EXPERIMENTAL

The method of discontinuous non-denaturing gel electrophoresis, with buffers of Bistris/HCl, Bistris/phosphate and Bistris/Tes, has been described in detail (Van Patten *et al.*, 1986). The preparation of pure rabbit skeletal-muscle inhibitor protein and pure bovine heart cyclic-AMP-dependent protein kinase catalytic subunit, the assay of these two proteins, and other methodologies, were as presented previously (Whitehouse & Walsh, 1983*a*; Whitehouse *et al.*, 1983; Van Patten *et al.*, 1986; Kinzel *et al.*, 1987).

RESULTS

In accord with the past data of Kinzel *et al.* (1987), the chromatography of pure catalytic subunit from bovine heart type II cyclic AMP-dependent protein kinase on CM-cellulose eluted with a shallow gradient led to the separation of two species of enzyme (Fig. 1). In order of elution, these are designated C_A and C_B (Kinzel *et al.*, 1987). The amount of C_A in different pure preparations of the cardiac enzyme is in the range 10–30% (see also Fig. 2 in Kinzel *et al.*, 1987). (A reason for some variability among preparations in the amounts of C_A and C_B present in the pure enzyme is the choice of fractions that are pooled at various steps during the enzyme preparation, in particular at the penultimate CM-cellulose chromatography fractionation.) The presence of C_A and C_B has been demonstrated in addition in preparations of protein kinase from rabbit and rat skeletal-muscle and pig heart (Kinzel *et al.*, 1987; S. M. Van Patten, unpublished work).

The individual fractions obtained by CM-cellulose chromatography were examined for complex-formation with the inhibitor protein by using the non-denaturing gel-electrophoresis system we have previously detailed (Van Patten *et al.*, 1986). As illustrated (Fig. 1 inset), C_A and C_B each gave rise to a single species of complex, with C_A giving the complex previously designated $CI_{0.30}$, and C_B the $CI_{0.26}$ species. The C_A/C_B ratio given by the CM-cellulose chromatography (approx. 15% C_A), is similar to the $CI_{0.30}/CI_{0.26}$ ratio that we previously obtained by electrophoresis of unfractionated protein kinase (approx. 30% C_A; Van Patten *et al.*, 1986; see also Fig. 3 and comment above on variability in the amounts of C_A and C_B). These results demonstrate

‡To whom correspondence should be addressed.

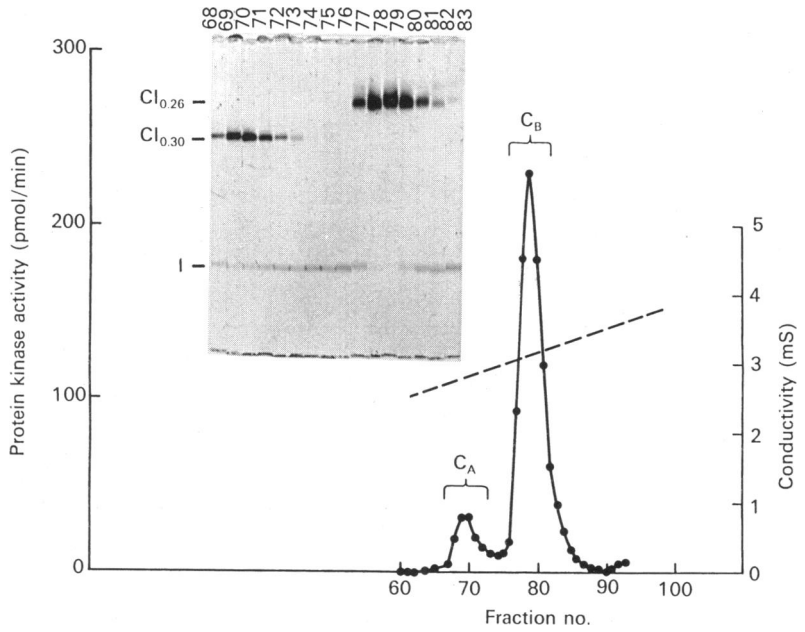

Fig. 1. CM-cellulose chromatography of pure bovine heart cyclic AMP-dependent protein kinase

A 2 ml (1.25 mg) portion of bovine heart cyclic AMP-dependent protein kinase catalytic subunit, equilibrated in 5 mM-potassium phosphate buffer, pH 6.0, containing 2 mM-EDTA and 15 mM-2-mercaptoethanol was applied to a 0.9 cm × 60 cm column of CM-cellulose (CM-52; Whatman) in this buffer. The column was eluted with a linear gradient of 5–120 mM-potassium phosphate buffer, pH 6.0, containing 2 mM-EDTA and 15 mM-2-mercaptoethanol. The flow rate was 10 ml/h and the fraction size was 3.5 ml. Protein kinase activity was measured as described previously (Whitehouse & Walsh, 1983*b*) with mixed histone (Sigma IIA) as substrate. Inset. To prepare column fractions for gel electrophoresis, either 2 ml of fractions 68–75 was concentrated to 70 μl by centrifugation at 5000 *g* for 2 h in Centricon-10 concentrators (Amicon), or 0.5 ml of fractions 76–83 was concentrated to 120 μl by centrifugation with a similar procedure for 20 min. To each of these samples was added 20 μl of pure inhibitor protein (3.1 munits) and the appropriate volume of 5 × -concentrated sample buffer. Each of these fractions was then electrophoresed on the non-denaturing discontinuous gel system that we have described in detail in Van Patten *et al.* (1986), with 100 μM-ATP and 400 μM-$MgCl_2$ in the upper reservoir buffer. Notated on the gel are the positions of migration of inhibitor protein (I), and the $CI_{0.26}$ and $CI_{0.30}$ complexes that are detected with unfractionated pure catalytic subunit (Van Patten *et al.*, 1986). Uncomplexed catalytic subunit, being cationic, does not enter the non-denaturing gel. In addition to $CI_{0.26}$ and $CI_{0.30}$, a third very minor form of complex ($CI_{0.22}$) was reported previously (Van Patten *et al.*, 1986). A similarly located minor complex is observable in fraction nos. 82 and 83.

that our previous observations of multiple inhibitor protein–catalytic subunit complexes arise as a consequence of multiple species of protein kinase catalytic subunit.

Characteristics of the CI complexes

$MgATP^{2-}$-dependence. Previous kinetic studies (Whitehouse & Walsh, 1983*a*) demonstrated that maximum interaction of the inhibitor protein with the catalytic subunit requires the presence of $MgATP^{2-}$, a property compatible with the established ordered reaction mechanism of the protein kinase (Whitehouse *et al.*, 1983) and with the binding of the inhibitor protein to the protein substrate site (Ashby & Walsh, 1972; Reed *et al.*, 1987). Physicochemical studies using the non-denaturing gel electrophoresis system also indicated that $MgATP^{2-}$ was required for complex-formation, but the interpretation of these latter data, with respect to each of the complexes, was complicated by the presence of both C_A and C_B in the protein kinase preparation and the potential alteration of the concentration of Mg^{2+} and ATP during electrophoresis (Van Patten *et al.*, 1986). The $MgATP^{2-}$requirement has now been evaluated for each form of the complex separately. These data are presented in Fig. 2, which depicts the formation of C_AI and C_BI in the presence and in the absence of $MgATP^{2-}$ at two concentrations of inhibitor protein, the first with catalytic subunit in approx. 50% excess, the second with a 2-fold excess of inhibitor protein. As is illustrated by these data, both C_AI and C_BI formation exhibited a strong $MgATP^{2-}$-dependence. Under the condition of catalytic subunit excess, no complex-formation was detectable in the absence of added nucleotide. [Under similar conditions $MgATP^{2-}$-dependence of C_AI and C_BI complex-formation was also observed with the two species added together (results not shown).] A small degree of $MgATP^{2-}$-independence is seen with C_BI when excess inhibitor protein is present, whereas C_AI formation under these conditions was fully $MgATP^{2-}$-dependent. Previously with the unfractionated enzyme (Van Patten *et al.*, 1986) in the presence of excess inhibitor protein considerable $MgATP^{2-}$-independent complex-formation was seen with both C_A and C_B, but the reason for these differences now seen with the separated C_A and C_B proteins is unclear. Most probably, under physiological conditions with the concentrations of inhibitor protein and catalytic subunit present in the cell, complex-formation is likely to be fully $MgATP^{2-}$-dependent.

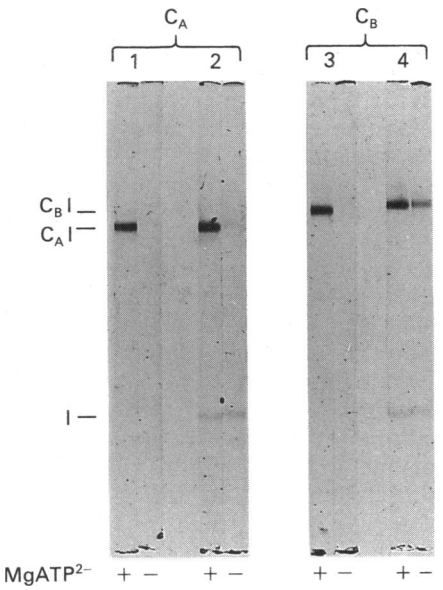

Fig. 2. $MgATP^{2-}$-dependence of C_AI and C_BI formation

In each of the four pairs of gels, the left-hand gel was run in the presence of 100 μM-ATP and 400 μM-$MgCl_2$ in the upper reservoir. In the right-hand gel of each pair, $MgATP^{2-}$ was absent. Both gels of each pair had the same protein sample applied, as follows: pair 1, 160 pmol of C_A and 106 pmol of inhibitor protein; pair 2, 160 pmol of C_A and 318 pmol of inhibitor protein; pair 3, 160 pmol of C_B and 106 pmol of inhibitor protein; pair 4, 160 pmol of C_B and 318 pmol of inhibitor protein. All other conditions of electrophoresis were as described in Van Patten *et al.* (1986).

Competitive interaction of C_A and C_B with the inhibitor protein. To examine the relative affinities of C_A and C_B for the inhibitor protein, the extent of C_AI and C_BI formation was determined as a function of various amounts of each species of catalytic subunit with a fixed and limiting amount of inhibitor protein. The non-denaturing gels of these experiments are depicted in Fig. 3. Next to each gel are expressed the ratios of C_A and C_B added and the ratios of the amounts of C_AI and C_BI formed, the latter being determined from scanning of the stained gels. Two controls are also shown. The first (C-1) illustrates for one of the conditions, the maximum (and complete) formation of the two complexes in the presence of excess inhibitor protein. This control provides evidence that, in the experimental gels (1–9), complex formation is limited by the amount of inhibitor protein present and, thus, these conditions are suitable for examination of the relative affinities of C_A and C_B. The second control gel of Fig. 3 (C-2) depicts the typical pattern of complex-formation obtained with unfractionated pure catalytic subunit. As illustrated by the data of Fig. 3, with the amount of C_A fixed and the amount of C_B being increased from one-third of the amount of C_A to 3-fold higher than that of C_A (gels 1–5), there was a proportional increase in C_BI formation and an equivalent decrease in C_AI. A similar observation was also obtained with a fixed amount of C_B and variation in the amount of C_A (gels 6–9). Total complex-formation (i.e. C_AI+C_BI) was similar in all gels (1–9) and limited by the amount of inhibitor protein present. Under all conditions the C_AI/C_BI ratio was directly equal, within experimental error, to the ratio of C_A and C_B added. These data indicate that C_A and C_B have equal affinities

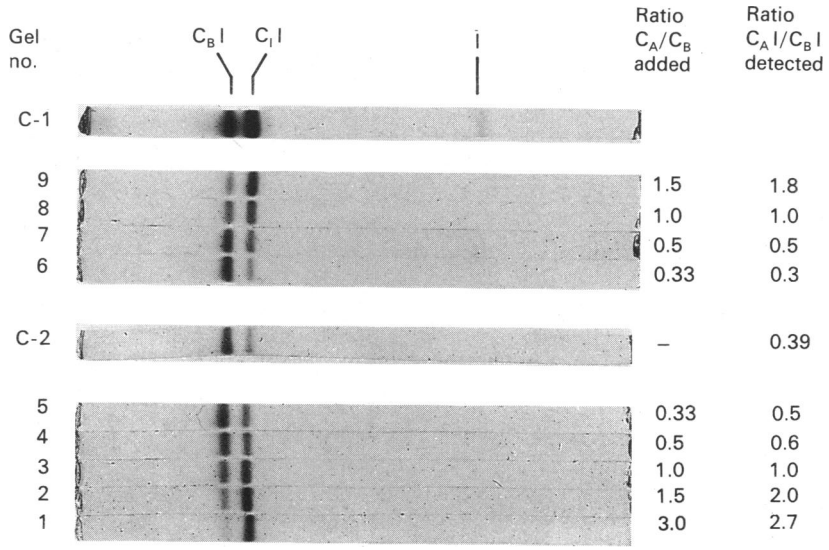

Fig. 3. Competitive interaction of C_A and C_B with the inhibitor protein

Samples applied to gels 1–5 contained 161 pmol of C_A, 107 pmol of inhibitor protein and 53.6 pmol (gel 1), 107 pmol (gel 2), 161 pmol (gel 3), 322 pmol (gel 4) or 482 pmol (gel 5) of C_B. Samples applied to gels 6–9 contained 161 pmol of C_B, 107 pmol of inhibitor protein and 53.6 pmol (gel 6), 80.4 pmol (gel 7), 161 pmol (gel 8) or 241 pmol (gel 9) of C_A. The sample applied to control gel C-1 contained 241 pmol of C_A, 161 pmol of C_B and 536 pmol of inhibitor protein (i.e. the same as for gel 9 but with 5 times as much inhibitor protein). The sample applied to control gel C-2 contained 429 pmol of purified catalytic subunit (before separation of C_A and C_B) plus 107 pmol of inhibitor protein. All gels were run with 40 μM-ATP and 400 μM-$MgCl_2$ in the upper reservoir. All other conditions of electrophoresis were as described in Van Patten *et al.* (1986). After staining by Coomassie Blue, the ratio of complex-formation was quantified by scanning the gels at 595 nm with a Cary 210 spectrophotometer with the peak areas determined by direct integration.

for the inhibitor protein. This conclusion has been confirmed by an examination of the titration of catalytic activity of C_A and C_B with increasing concentrations of inhibitor protein. As illustrated (Fig. 4), both are titrated equally.

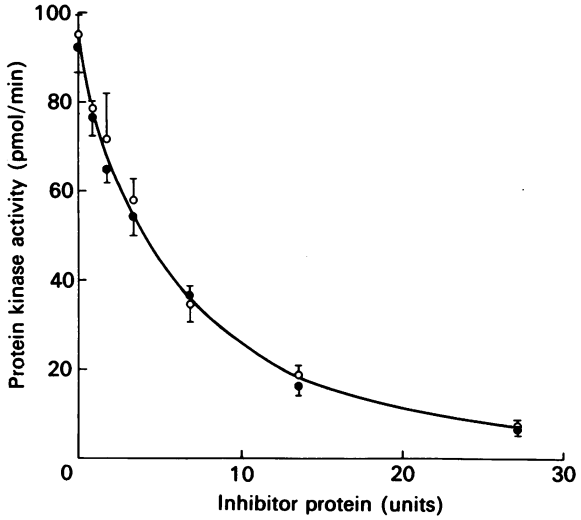

Fig. 4. Titration of C_A and C_B activity by inhibitor protein

C_A (●) and C_B (○) were diluted in 10 mM-Mes buffer, pH 6.8, containing 2 mg of bovine serum albumin/ml to equal concentrations and then assayed for protein kinase activity with increasing concentrations of inhibitor protein by using the assay procedure described previously (Whitehouse & Walsh, 1983*b*) with mixed histone (Sigma IIA) as substrate. Each point represents a triplicate determination.

The data of Figs. 3 and 4 suggested that C_A and C_B would probably exhibit equivalent protein substrate specificities, since it has been established that the inhibitor protein binds to the protein kinase at the protein substrate site with at least several of the same equivalent recognition signals (Scott *et al.*, 1985, 1986; Cheng *et al.*, 1986). To assess this further, the phosphorylation of phosphorylase kinase as catalysed by isolated C_A and C_B was examined. This substrate for the protein kinase provides the opportunity not only of examining the relative rates of phosphorylation of an individual pure substrate, but also of contrasting the separate rates of phosphorylation of its α- and β-subunits, each of which contains substrate sites for the protein kinase with distinct peptide recognition sequences (Cohen *et al.*, 1975). Phosphorylation of phosphorylase kinase was examined under two distinct buffer conditions that alter the available phosphorylation sites in the α-subunit (Ramachandran *et al.*, 1987). The results of these experiments are presented in Fig. 5 and illustrate that C_A and C_B exhibit identical affinity for phosphorylase kinase. These data suggest that the function of these two forms of protein kinase catalytic subunit is other than a recognition of distinct target substrates.

DISCUSSION

The data reported in the present paper elucidate why it had previously been observed that the interaction of pure inhibitor protein with pure cyclic AMP-dependent protein kinase catalytic subunit led to the formation of multiple complexes (Van Patten *et al.*, 1986). The reason for the existence of multiple forms of catalytic subunit is not, at this time, apparent, but the presence of multiple

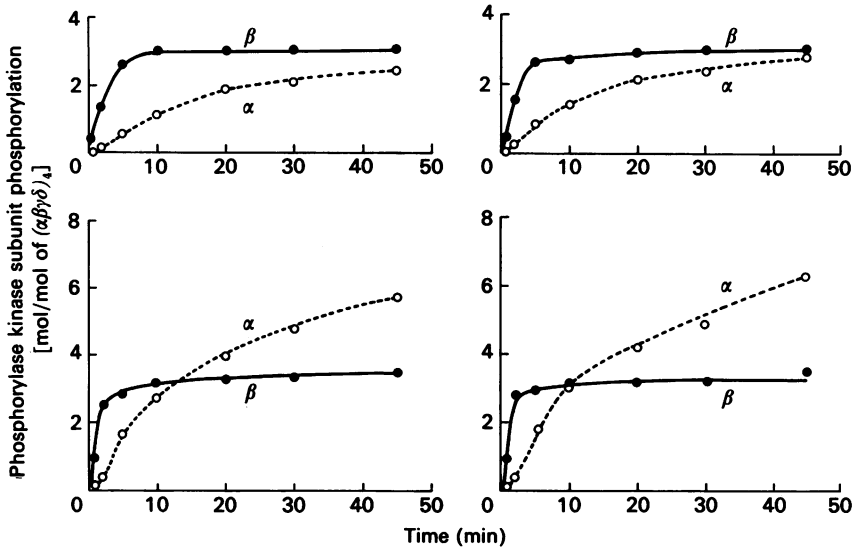

Fig. 5. Comparison of phosphorylase kinase phosphorylation by C_A and C_B

Equal activities, with respect to histone phosphorylation, of C_A (left-hand panels) and C_B (right-hand panels) were used to examine the rates of phosphorylation of the α-subunits (○) and β-subunits (●) of skeletal-muscle phosphorylase kinase. The phosphorylation of phosphorylase kinase (375 μg) by 1 munit of each form of catalytic subunit was examined in reaction mixtures of either (upper panels) 34 mM-glycerophosphate/6 mM-Hepes buffer, pH 6.8, containing 50 mM NaF, or (lower panels) 50 mM-Hepes buffer, pH 6.8, plus (in both reaction mixtures) 3.2 mM-$MgCl_2$, 112 μM-[γ-^{32}P]ATP, 2.7 mM-2-mercaptoethanol, 0.3 mM-EGTA, 1.4 mM-EDTA, 10% (w/v) sucrose and 0.5 mg of bovine serum albumin/ml. These two conditions lead to different patterns of α-subunit phosphorylation (Ramachandran *et al.*, 1987). All other conditions of the reaction and the method utilized to examine subunit phosphorylation were as described previously by Ramachandran *et al.* (1987).

species is now evident from several investigations and is likely to have important physiological ramifications. Recently, two isoforms of the catalytic subunit, designated C_α and C_β, have been identified from cDNA clones (Uhler *et al.*, 1986*a*,*b*; Showers & Maurer, 1986). The possibility exists that the products of these distinct genes correspond to C_A and C_B. Amino acid sequence data for C_α and C_β suggests that C_α corresponds to the predominant species sequenced from bovine heart, i.e. C_B (Shoji *et al.*, 1981). However, as seen from data presented here and in Van Patten *et al.* (1986), the electrophoretic mobilities in the presence of the inhibitor protein indicate that C_A is more negative than C_B, as would also be predicted by their respective elution from CM-cellulose, whereas calculations, based on the deduced amino acid sequences from the clones, suggest that the C_β-gene product would be less negative than that from the C_α gene. A more definitive comparison awaits either the production of specific antibodies or the availability of the C_β-gene product in sufficient amounts. However, what is apparent from the current studies is that C_A and C_B have identical reactivities towards the inhibitor protein, and have very similar, if not identical, substrate specificities; thus their different functions await elucidation.

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REFERENCES

Ashby, C. D. & Walsh, D. A. (1972) J. Biol. Chem. **247**, 6637–6642

Bechtel, P. J., Beavo, J. A. & Krebs, E. G. (1977) J. Biol. Chem. **252**, 2691–2697

Chen, L.-J. & Walsh, D. A. (1971) Biochemistry **10**, 3614–3621

Cheng, H. C., Van Patten, S. M., Smith, A. J. & Walsh, D. A. (1985) Biochem. J. **231**, 655–661

Cheng, H. C., Kemp, B. E., Pearson, R. B., Smith. A. J., Misconi, L., Van Patten, S. M. & Walsh, D. A. (1986) J. Biol. Chem. **261**, 989–992

Cohen, P., Watson, D. C. & Dixon, G. H. (1975) Eur. J. Biochem. **51**, 79–92

Kinzel, V., Hotz, A., König, N., Gagelmann, M., Pyerin, W., Reed, J., Köbler, D., Hofmann, F., Obst, C., Gensheimer, H. P., Goldblatt, D. & Shaltiel, S. (1987) Arch. Biochem. Biophys. **253**, 341–349

McPherson, J. M., Whitehouse, S. & Walsh, D. A. (1979) Biochemistry **18**, 4835–4845

Ramachandran, C., Goris, J., Waelkens, E., Merlevede, W. & Walsh, D. A. (1987) J. Biol. Chem. **262**, 3210–3218

Reed, J., Gagelmann, M. & Kinzel, V. (1983) Arch. Biochem. Biophys. **222**, 276–284

Reed, J., Kinzel, V., Cheng, H. C. & Walsh, D. A. (1987) Biochemistry **26**, 7641–7647

Scott, J. D., Fischer, E. H., Demaille, J. G. & Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4379–4383

Scott, J. D., Glaccum, M. B., Fischer, E. H. & Krebs, E. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1613–1616

Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, D. A., Neurath, H., Long, G. L., Demaille, J. G., Fischer, E. H. & Titani, K. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 848–851.

Showers, M. O. & Maurer, R. A. (1986) J. Biol. Chem. **261**, 16288–16291

Uhler, M. D., Carmichael, D. F., Lee, D. C., Chrivia, J. C., Krebs, E. G. & McKnight, G. S. (1986*a*) Proc. Natl. Acad. Sci. U.S.A. **83**, 1300–1304

Uhler, M. D., Chrivia, J. C. & McKnight, G. S. (1986*b*) J. Biol. Chem. **261**, 15360–15363

Van Patten, S. M., Fletcher, W. H. & Walsh, D. A. (1986) J. Biol. Chem. **261**, 5514–5523

Whitehouse, S. & Walsh, D. A. (1983*a*) J. Biol. Chem. **258**, 3682–3692

Whitehouse, S. & Walsh, D. A. (1983*b*) Methods Enzymol. **99**, 80–93

Whitehouse, S., Feramisco, J. R., Casnellie, J. E., Krebs, E. G. & Walsh, D. A. (1983) J. Biol. Chem. **258**, 3693–3701

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