Ala^{335} is essential for high-affinity cAMP-binding of both sites A and B of cAMP-dependent protein kinase type I

Michaele Zorn[a,b], Kari E. Fladmark[c], Dagfinn Øgreid[c], Bernd Jastorff[b], Stein O. Døskeland[d], Wolfgang R.G. Dostmann[a,*]

[a]*Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Str. 29, 80802 München, Germany*
[b]*Institut für Organische Chemie, Universität Bremen, Leobener Str., NW 2, 28359 Bremen, Germany*
[c]*Center of Molecular Medicine, Haukeland Hospital, niversity of Bergen, 5021 Bergen, Norway*
[d]*Institute of Anatomy and Cell Biology, University of Bergen, Årstadveien 19, 5009 Bergen, Norway*

Received 14 February 1995; revised version received 5 March 1995

Abstract A single amino acid substitution ($Ala^{335}Asp$) in cAMP binding site B of the regulatory subunit of cAMP-dependent protein kinase type I was sufficient to abolish high affinity cAMP binding for both cAMP binding sites A and B. Furthermore, the $Ala^{335}Asp$ mutation increased the activation constant for cAMP of the mutant holoenzyme 30-fold and also enhanced the rate of holoenzyme formation. Thus, the substitution was responsible for the dominant negative phenotype of the enzyme. Activation of mutant holoenzyme with site-selective cAMP analogs indicated that the enzyme dissociated through binding to site A only. Our results provide evidence that Ala^{335} is an essential residue for high affinity cAMP binding of both sites as well as for the functional integrity of the enzyme.

Key words: Cyclic AMP-dependent protein kinase; cAMP analog; Site-directed mutagenesis

1. Introduction

The holoenzyme complex of the cAMP-dependent protein kinase (R_2C_2) consists of a regulatory subunit dimer (R_2) and two catalytic subunits (C). Binding of cAMP to the regulatory subunits causes the complex to dissociate and subsequently release the active catalytic subunit (for reviews see [1,2]). Each regulatory subunit monomer consists of two in-tandem cAMP binding sites at the C-terminal end of the protein [3]. In addition, the N-terminus hosts the dimerisation region and the 'hinge' region responsible for complexing the catalytic subunit [3,4]. The two cAMP binding sites are known to bind cAMP and cAMP analogs with different affinities [5,6]. Sequence alignment of the cAMP binding domains revealed the homology to the catabolite gene activator protein (CAP), and on the basis of the 2.5 Å crystal structure of CAP a three-dimensional model for each cAMP binding site has been constructed [7,8]. The validity of this model as a general framework for the folding of the polypeptide chain in both cAMP binding sites is confirmed by photoaffinity labeling [9] and by analyzing mutant forms of the R^I subunit defective in their cAMP binding sites [10–12]. The general features of the cAMP binding sites (A and B) include an invariant Arg (209 and 333) which binds to the phosphate moiety of cAMP, and an invariant Glu (200 and 324) that hydrogen bonds to the 2'-OH of the ribose. Additional invariant residues are known but only Ala^{334} in site B has been shown to interact with the adenine moiety since alteration of this residue changed the affinity and specificity for cGMP [13]. Recently, a sub-clone of the rat myelocytic leukemia cell line (IPC-81) has been found to harbor a heterozygously expressed point mutation [14]. This mutated clone was selected by treatment with 8-CPT-cAMP and identified as Ala-to-Asp at the invariant position 335 [14]. In order to study the effects of this mutation on ligand binding and holoenzyme activation we used recombinant protein expression techniques to obtain large quantities of pure recombinant $Ala^{335}Asp$ mutant protein.

2. Materials and methods

2.1. Mutagenesis

The mutagenesis was performed by PCR 'overlap extension' [15]. Primary PCR reaction mixtures (100 µl) contained: 50 mM Tris, pH 9.0, 50 mM KCl, 7 mM $MgCl_2$, 0.125 mM dNTPs, 0.2 mg/ml BSA, 16 mM $(NH_4)_2SO_4$, 20 mM of primer A (5'-CGGCCCCGTGCAGATACCG-TGGTG-3') and B (5'-CTCGCATGCTCAGACAGACAGGGACA-CGA-3') or C (5'-ACCACGGTATCTGCAC-3') and D (5'-GATTA-CGAATTCCCCGGATCCGT-3'), 1 ng DNA (full-length pUC-7/R^I cDNA clone) [16] and 0.1 U Super Taq-polymerase (HT Biotech). The amplified DNA products were purified and incubated with Klenow fragment before extraction with Quiaex beads (Quiagen). A subsequent PCR reaction contained a mixture of the two primary PCR products and primers B and D. The final PCR product was purified, cut with *Eco*RI and *Sph*I, ligated into pUC-7 and transformed into electrocompetent *E. coli* JM109 cells. Positive clones (pUC-7/A335D-R^I) were selected and the amplified insert DNA sequenced according to the method described by Sanger [17].

2.2. Expression and purification of recombinant mutant and wild-type R^I subunits

4 l of LB broth (100 µg/ml ampicillin) were inoculated with *E. coli* JM109 (pUC-7/A335D-R^I). Cells were grown at 37°C for 24 h and the bacterial pellet subsequently resuspended in buffer A (10 mM potassium phosphate, pH 7.0, 15 mM β-mercaptoethanol, 2 mM EDTA, 2 mM benzamidine, 0.1 M NaCl, 100 mg/ml PMSF, 50 mg/ml SBTI, 100 mg/ml TPCK and 50 mg/ml TLCK) and passed twice through a French pressure cell at 4°C. The homogenate was centrifuged and the protein purified to homogeneity on DEAE- and cAMP-agarose (8-AEA-cAMP) affinity chromatography. Holoenzyme was formed as described earlier [18] by dialysis of the R^I subunit with a 10% molar excess of C subunit. After dialysis excess of C subunit was removed with CM-Sepharose. Catalytic activity was assayed by a coupled spectrophotometric method [19]. The synthetic peptide L-R-R-N-S-I was used as substrate [18]. The rate of holoenzyme formation was determined by removing aliquots at indicated time points and comparing the remaining activity to wild-type enzyme. The expression and purification of wild-type R^I subunit was as described in [16].

2.3. cAMP binding assays

K_D for wild-type and mutant R^I subunits and their holoenzymes were

*Corresponding author. Fax: (49) (3849) 3261.

SSDI 0014-5793(95)00261-8

determined by modification of a method described earlier [20]. Proteins were incubated in buffer A (50 mM MES, pH 6.9, 0.4 mM EGTA, 1 mM Mg-acetate, 10 mM NaCl, 10 mM DTT and 0.5 mg/ml BSA) with varying concentrations of [^{3}H]cAMP (Amersham). After 30 min incubation at 30°C aliquots were precipitated in 3 ml of ice-cold 95% ammoniumsulfate (with 10 mM HEPES-NaOH, pH 7.0, 2 mM EDTA) and filtered over 0.45 μm pore size nitrocellulose filters. To distinguish the two kinetically different cAMP binding sites [^{3}H]cAMP bound to site A was selectively displaced by an excess of unlabeled cAMP during a 5–6 h incubation at 0°C as previously described [20].

cAMP dissociation rates (k_{off}) were measured in the absence of C-subunit. The individual R^I-subunit proteins (50 nM) were saturated with [^{3}H]cAMP (1 μM) during 30 min incubation at 30°C followed by 30 min at 0°C in a final volume of 100 μl binding buffer (with 60 mM HEPES-NaOH, pH 7.0, 60 mM EDTA, 20 mM DTT, 0.5 mg/ml BSA). Aliquots were added to dissociation buffer (15 mM HEPES-NaOH, pH 7.0, 5 mM EDTA, 0.3 mM EGTA, 20 mM DTT, 0.5 mg/ml BSA, 4 M NaCl and 1 mM cAMP). At designated time points, aliquots were removed, and [^{3}H]cAMP that remained bound to R^I subunit was measured by the ammonium sulfate filtration assay as described above.

2.4. *Apparent activation constants ($K_{a,app}$) for cAMP and cAMP analogs*

Mutant and wild-type holoenzymes (10 nM) were incubated with the synthetic peptide L-R-R-N-S-I (40 μM), 0.1 mM [γ-^{32}P]ATP (Amersham; specific activity 150 cpm/pmol) and various concentrations of cAMP or adenine-modified cAMP analogs. The phosphotransferase reaction was carried out for 2 min at 30°C according to a method described previously [21]. Reactions were started by addition of the holoenzymes and terminated after 2 min by spotting aliquots on phospho-cellulose strips and subsequently washed in 75 mM phosphoric acid.

3. Results

3.1. *Purification and cAMP-binding*

Expression and subsequent purification of the mutant Ala^{335}Asp R^I subunit yielded a soluble and stable protein of 45 kDa as judged from SDS-PAGE (data not shown). No proteolytic degradation, which is prone to occur at the hinge region, was observed for the duration of the experiments. Since the mutant R^I subunit was obtained from cAMP-agarose at the last purification step it obviously retained some cAMP binding capability. To further investigate the cAMP binding properties

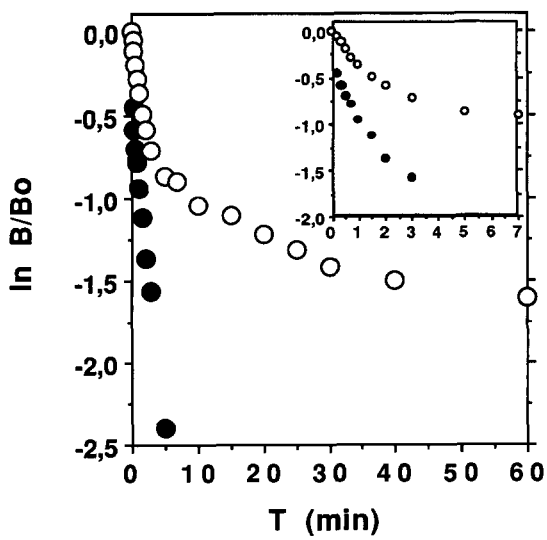

Fig. 1. Rate of exchange of [^{3}H]cAMP bound wild-type (○) and Ala^{335}Asp regulatory subunit (●). The inset shows the initial rate enlarged.

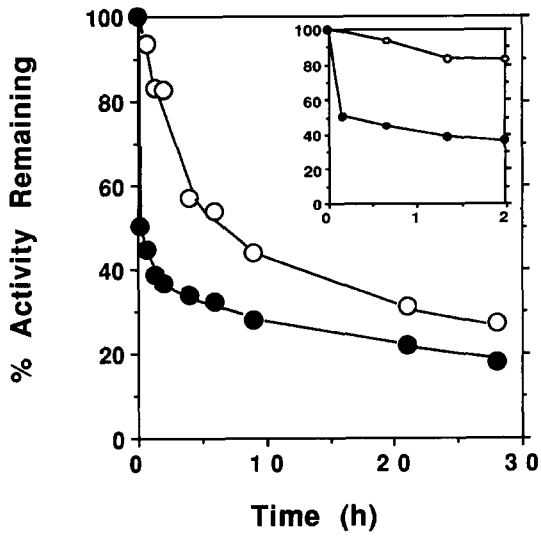

Fig. 2. Rate of holoenzyme formation in the presence of MgATP for wild type (○) and mutant proteins (●).

we determined the binding and dissociation constants and compared them to wild-type R^I subunit. As shown in Fig. 1 and Table 1 the two cAMP binding sites in the wild-type R^I subunit are readily distinguished by their cAMP dissociation rates. The rapid dissociation (k_{off} = 1.0 min^{-1}) represents the exchange of cAMP bound to site A while the slower dissociation (k_{off} = 0.0167 min^{-1}) is the exchange from site B [22]. Dissociation rates were measured with high salt in the buffer, a condition known to emphasize biphasic exchange [23]. In contrast, the cAMP dissociation rate of the mutant R^I subunit was monophasic and rapid (k_{off} = 1.2 min^{-1}, see inset of Fig. 1), indicating one functional class of 'binding site' per monomer.

Equilibrium cAMP binding studies revealed that the wild-type protein bound 2 mol cAMP/mol subunit with K_D values of 10 nM and 30 nM for binding site A and B, respectively. In contrast, the Ala^{335}Asp mutant bound 1 mol cAMP/mol subunit with an apparent K_D of 490 nM (Table 1). Thus, high affinity binding to the mutated site was not only abolished, but the mutation transmitted a change in binding interaction to the non-mutated binding site A as well. The corresponding mutant protein extracted from IPC-81 cells [14] showed a slightly higher affinity for site A compared to the data obtained in this

Table 1
Kinetic constants of wild-type and Ala^{335}/Asp mutant proteins

	Wild-type	Ala 335/Asp
R^I subunit		
Rate constant, k_{off} (min^{-1})	site A: 1.0 site B: 0.0167	1.2
Dissociation constant, K_D (nM)	22	490
Binding stochiometry	2	1
Holoenzyme		
Activation constant, $K_{a,app}$ (nM)	170	5,000
Dissociation constant, K_D (nM)	165	2,800
Binding stochiometry	2	1

The kinetic constants were determined as described in section 2.

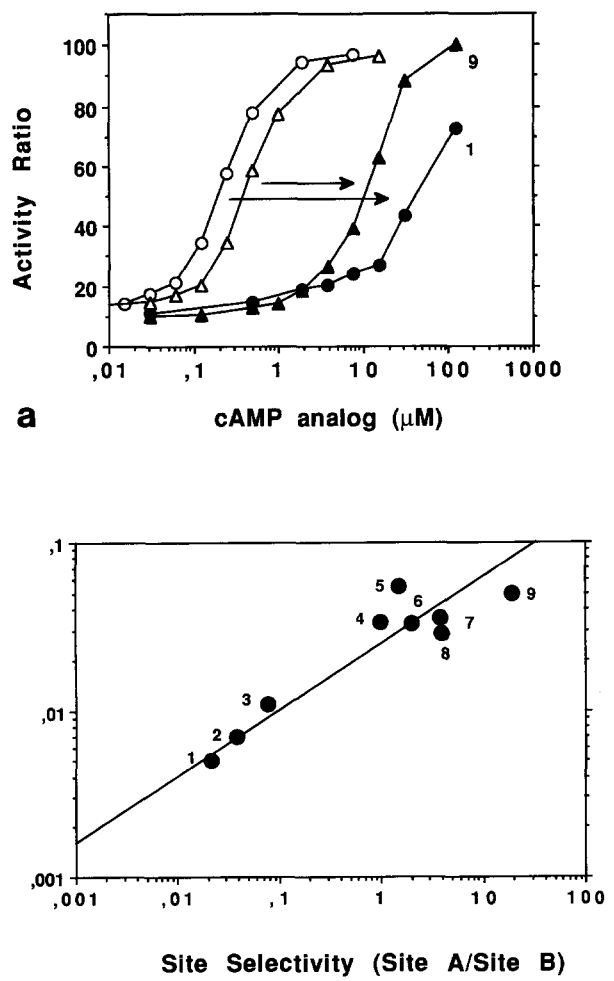

Fig. 3. (a) The ability of 8-Methylamino-cAMP (1, circles) and 6-benzoyl-cAMP (9, triangles) to activate the wild-type (open symbols) and mutant holoenzyme (filled symbols). The shift for each analog is indicated by arrows. (b) The relative ability of cAMP analogs (●) to activate the $Ala^{335}Asp$ mutant and wild-type holoenzymes ($K_{a,app}$ cAKI/$K_{a,app}$ cAK-R^1aA335D) as a function of the A/B binding site selectivity for these cAMP analogs. The numbers in the double reciprocal plot indicate the individual cAMP analogs (see Table 2).

study. This apparent discrepancy can be explained by (i) the different species as sources of the proteins, (ii) the higher concentration of recombinant enzyme, or (iii) a possible macromolecular aggregation of the recombinant protein. When measuring equilibrium cAMP binding with mutant holoenzyme (see section 3.2) the binding constant (K_D = 2.8 μM) was increased even further (Table 1).

3.2. Holoenzyme formation and activation

In order to determine whether mutant R^I subunit retained the ability to interact with the C subunit, $Ala^{335}Asp$ R^I subunit was dialyzed in the presence of a 10% excess of C subunit under conditions that would typically lead to the formation of holoenzyme [18]. Holoenzyme formation was measured in the presence of MgATP which previously had been shown to strongly increase the rate at which wild-type R^I subunit associates with C subunit [24]. Under these conditions, the mutant R^I subunit formed holoenzyme much faster than the wild-type protein (Fig. 2). In fact holoenzyme formation occured nearly spontanously and did not appear to require dialysis for displacement of cAMP (data not shown).

The activation of holoenzyme as a function of cAMP concentration was tested using the phosphotransferas assay. The apparent activation constant ($K_{a,app}$) was measured in the presence of MgATP, which is known to lower the affinity for cAMP and thereby the activation potential of the holoenzyme [10]. The cAMP concentration needed for half-maximal activation ($K_{a,app}$) of the mutant holoenzyme was 5 μM in comparison to 170 nM for the wild-type protein (Table 2). Thus, by changing Ala^{335} to Asp the $K_{a,app}$ value increased 30-fold.

3.3. cAMP-analog specificity

Since the two cAMP binding sites in the native R^I subunit are known to preferentially bind different adenine modified analogs of cAMP [5], a total of nine adenine-modified analogs with a broad range of site B to site A selectivities were studied (Table 2). The ability of these analogs to activate the wild-type and mutant holoenzyme is shown in Fig. 3a for 8-methylamino-cAMP (no. 1) and 6-benzoyl-cAMP (no. 9). 8-Methylamino-cAMP, which binds preferentially to site B, was nearly as effective as cAMP in activating the wild-type holoenzyme and was even more effective compared to 6-Benzoyl-cAMP (no. 9) which preferentially binds to site A. However half-maximal activation of the mutant protein by the B-selective 8-Methylamino-cAMP shifted to higher concentrations as did activation by cAMP or A-selective analogs like 6-Benzoyl-cAMP. Fig. 3b shows the ability of these cAMP analogs to activate the mutant holoenzyme relative to the wild type protein compared to their selectivity for site A/B. All analogs showed a shift to higher concentrations for their ability to activate the mutant enzyme (Table 2). In addition, analogs preferring site B were relatively weaker as activators of the mutant holoenzyme compared to A-selective analogs (Fig. 3b).

4. Discussion

cAMP-dependent protein kinase requires two functional cAMP binding sites for allosteric regulation. Kinetic evidence [22] as well as results from cAMP binding site mutants [10] and cAMP analogs [25,26] have indicated that kinase activation is mediated via a sequential kinetic mechanism. First, cAMP

Table 2
Site selectivity (R^I) and apparent activation constants of adenine-modified cAMP analogs

No.	cAMP analog	Site selectivity	Activation constants, $K_{a,app}$ (μM)	
		$R^{I\,A/B}$	Wild-type	Mutant
1	8-Methylamino-cAMP	1/47	0.20	40
2	8-Amino-cAMP	1/28	0.15	20
3	2-Chloro-cAMP	1/13	0.28	25
4	cAMP	1.0	0.17	5
5	7-Deaza-cAMP	1.5	0.55	10
6	8-*p*-Chlorophenylthio-cAMP	2.0	0.04	1.2
7	N^6-Benzyl-cAMP	3.9	0.31	9
8	N^1-Oxid-cAMP	4.0	0.50	17
9	N^6-Benzoyl-cAMP	19	0.50	10

The cAMP analogs are listed according to their $R^{I\,A/B}$ selectivity [5]. Apparent activations constants $K_{a,app}$ were determined as described in section 2.4.

binds at site B, which in turn mediates a conformational change which opens up site A. Only when both sites are occupied will the C subunit be released from the R^I subunit dimer. The strong coupling between the two binding sites is reflected by a positive cooperativity (n = 1.5–1.6) [27,28]. In the $Ala^{335}Asp$ R^I subunit we found that one high affinity cAMP binding site was apparently completely abolished, and we have shown that site B does not bind cAMP as a consequence of the mutation (Fig. 1, Table 1). Furthermore, site B is uncoupled from site A which leads to a drastically reduced binding affinity for site A. Dissociation experiments showing a monophasic and rapid off-rate for site A lent further support to the conclusion that cAMP binding to site B was abolished (Fig. 1).

Formation of mutant holoenzyme was extremely rapid (Fig. 2), as has been observed for a number of other cAMP-binding site mutants with reduced or completely abolished cAMP binding [10,11].

The combination of reduced affinity for site B and uncoupling of the two cAMP binding sites results in a conformation which is favorable for association with the C subunit. Consequently, those mutants display a '$K_{a,\,app}$-shift' which indicates that the mutant holoenzymes are more stable than wild-type enzyme. The activation and cAMP binding constants for the $Ala^{335}Asp$ mutant holoenzyme were similar (5 μM and 2.8 μM, respectively, Tables 1 and 2). The shift in the binding constants from 0.5 μM to 2.8 μM for site A in the R^I subunit and holoenzyme shows that (i) the catalytic subunit poses an additional barrier on cAMP binding to site A and (ii) binding of cAMP to site A is sufficient to activate the holoenzyme. Even in the holoenzyme complex the two sites are still uncoupled and site B does not impose a barrier on activation as is the case for wild-type holoenzyme. Activation studies with site selective cAMP analogs give further support to this hypothesis. The ability of site A selective analogs to preferentially activate mutant holoenzyme (Table 2, Fig. 3a,b) clearly indicates an activation mechanism of holoenzyme primarily through this cAMP binding site. However, the nature of the uncoupling in molecular terms is poorly understood. According to the CAP model [8] the side chain of Ala^{335} lies in close proximity to the adenine moiety of cAMP. The environment of the adenine binding pocket is mostly hydrophobic as has been shown by cAMP-analog mapping [5,29] and molecular modeling studies [13,30]. Particularly the Ala/Asp substitution, which introduces a charge in the adenine binding site, disrupts high affinity cAMP binding. Consequently, the mutated site B can not contribute to the positive cooperativity for binding to site A, hence the K_D measured for site A reflects the 'true' cAMP binding affinity in the R^I subunit. In conclusion, Ala^{335} in site B plays a major role in proper cAMP binding for the efficient and correct functioning of cAMP-dependent protein kinase.

Acknowledgements: We thank Nicole Koep and Iris Kühnert for excellent technical support. We also thank Dr. Susan S. Taylor for recombinant C-subunit. This work was supported in part by Deutsche Forschungsgemeinschaft Grant Do 329/2-2 (to W.R.G.D.) and the Norwegian Research Council (to D.Ø.).

References

[1] Francis, S.H. and Corbin, J.D. (1994) Annu. Rev. Physiol. 56, 237–272.
[2] Taylor, S.S., Buechler, J.A. and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005.
[3] Titani, K., Sasagawa, T., Ericsson, L.H., Kumar, S., Smith, S.B., Krebs, E.G. and Walsh, K.A. (1984) Biochemistry 23, 4193–4199.
[4] Potter, R.L. and Taylor, S.S. (1979) J. Biol. Chem. 254, 2413–2418.
[5] Øgreid, D., Ekanger, R., Suya, R. H., Miller, J. P. and Døskeland, S. O. (1989) Eur. J. Biochem. 181, 19–31.
[6] Rannels, S.R. and Corbin, J.D. (1981) J. Biol. Chem. 256, 7871–7876.
[7] Weber, I.T., and Steitz, T. A. (1987) J. Mol. Biol. 198, 311–326.
[8] Weber, I.T., Steitz, T.A., Bubis, J., and Taylor, S.S. (1987) Biochemistry 26, 343–351.
[9] Bubis, J. and Taylos, S.S. (1987) Biochemistry 26, 3478–3486.
[10] Neitzel, J.J., Dostmann, W.R.G. and Taylor, S.S. (1991) Biochemistry 30, 733–739.
[11] Øgreid, D., Døskeland, S.O., Gorman, K. and Steinberg, R.A. (1988) J. Biol. Chem. 263, 17397–17404.
[12] Woodford, T.A., Correll, L.A., McKnight, G.S. and Corbin, J.D. (1989) J. Biol. Chem. 264, 13321–13328.
[13] Shabb, J.B., Ng, L. and Corbin, J.D. (1990) J. Biol. Chem. 265, 16031–16034.
[14] Duprez, E., Gjertsen, B.T., Bernard, O., Lanotte, M. and Døskeland, S.O. (1993) J. Biol. Chem., 268, 8332–8340.
[15] Higuchi, R. (1990) in: PCR Protocols. A Guide to Methods and Applications (Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J. eds.) pp. 177–183, Academic Press, San Diego, CA.
[16] Saraswat, L.D., Filutowics, M. and Taylor, S.S. (1986) J. Biol. Chem. 261, 11091–11096.
[17] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[18] Dostmann, W.R.G. and Taylor, S.S. (1991) Biochemistry 30, 8710–8716.
[19] Cook, P.F., Neville, M.E., Vrana, K.E., Hartl, F.T. and Roskoski, J.R. (1982) Biochemistry 21, 5794–5799.
[20] Døskeland, S.O. and Øgreid, D. (1988) Methods Enzymol. 59, 147–150.
[21] Landgraf, W. and Hofmann, F. (1989) Eur. J. Biochem. 181, 643–650.
[22] Øgreid, D. and Døskeland, S.O. (1983) Biochemistry 22, 1686–1696.
[23] Døskeland, S.O. and Øgreid, D. (1984) J. Biol. Chem. 259, 2291–2301.
[24] Rangel-Aldao, R. and Rosen, O.M. (1977) J. Biol. Chem. 252, 7140–7145.
[25] Dostmann, W.R.G., Taylor, S.S., Genieser, H.-G., Jastorff, B., Døskeland, S.O. and Øgreid, D. (1990) J. Biol. Chem. 265, 10484–10491.
[26] Dostmann, W.R.G. and Taylor, S.S. (1991) Biochemistry 30, 8710–8716.
[27] Øgreid, D. and Døskeland, S.O. (1982), FEBS Lett. 150, 161–166.
[28] Bubis, J. and Taylor, S.S. (1987) Biochem. 26, 3478–3486.
[29] Jastorff, B., Hoppe, J. and Morr, M. (1979) Eur. J. Biochem. 101, 555–561.
[30] Dostmann, W.R.G., Störmann, R. and Jastorff B., (1990) Adv. Second Messenger Phosphoprotein Res. 24, 725.