Catalytic Subunit of Adenosine Cyclic 3′,5′ Monophosphate-Dependent Protein Kinase from Rat Muscle: Basic Properties and Factors Influencing the Activity

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Z. Naturforsch. **34 c,** 1186–1194 (1979); received June 25, 1979

Cyclic AMP-Dependent Protein Kinase, Catalytic Subunit, General Properties, Factors Influencing Activity, Rat Muscle

The catalytic (C) subunit of isoenzyme II of cyclic AMP-dependent protein kinase from rat muscle, purified to apparent homogeneity, was investigated for some of its basic properties and factors that influence its activity. The properties were found to resemble those of C subunits of both isoenzymes I and II from various other tissues of different species. This concerns the molecular weight estimate (about 37 k), ultraviolet light absorption spectrum, isoelectric point (pH 9.0 ± 0.2), the apparent K_M values for histone and protamine (for both substrates ≤ 10 μM) and the apparent K_M for cosubstrate ATP (3–4 μM). The enzyme is sensitive to the heat-stable inhibitory protein.

The enzyme activity is stable up to 40 °C but becomes destroyed at higher temperatures; the half life at 50 °C is slightly less than 1 min, and at ≥60 °C activity is nullified within 1 min. Activity is lost on freezing and thawing. Substrate histone does not improve temperature stability; cosubstrate ATP in absence of Mg^{2+} decreases, but in presence of Mg^{2+} increases stability. The optimum pH of enzyme activity is at pH 6.8 for histone and pH 7.8 for protamine phosphorylation. The enzyme becomes irreversibly inactivated at pH values below 5. For activity, the enzyme has absolute requirement for a divalent metal ion, highest activity is found in presence of Mg^{2+} or Co^{2+}; the optima are at 5–10 mM (half maximum at 0.9 mM) and 0.5–0.8 mM (half maximum at 0.25 mM) for Mg^{2+} and Co^{2+}, respectively. None of the various other metal ions tested were nearly as effective as these two. Ca^{2+} antagonized Mg^{2+}. A considerable effect on activity by ionic strength was observed. In dependence on the substrate used, activity is influenced strongly by some anions and zwitterions. *E. g.* in presence of acetate, histone phosphorylation is by a factor of 3–4 higher than in presence of sulfate, whereas protamine phosphorylation is only half that in presence of sulfate. Roughly the following order of enzyme activity in presence of the respective buffer system was found: MOPS (100%) ≥ TES (95%) ≅ HEPES > MES (65%) ≅ acetate ≫ ADA (20%) > phosphate (15%) > TRIS · maleate (7%).

Introduction

Proteins alter their structure and (in some cases) their function upon phosphorylation. This is utilized by cyclic AMP to translate its cell regulating message. The executing enzymes are cyclic AMP-dependent protein kinases (EC 2.7.1.37; ATP : protein phosphotransferases). These enzymes constitute, according to their behaviour on DEAE cellulose, two classes of isoenzymes, the type I and type II kinases, the ratio of which depending on species, tissue, and even cell type under consideration (reviewed [1 to 3]). They mediate their action via catalytic subunits (C) which are released from the holoenzyme on binding of cyclic AMP to regulatory subunits (R). On contrary to the latter, the C subunits are assumed to be identical in both types of isoenzymes [4–6]. However, it cannot be excluded at present that genetically determined distinct C subunits may exist (reviewed [3]). Although the principle nature of subunits and their interplay seems to be applicable generally, much work remains to be done to characterize subunits of different tissues in order to either confirm or change the present view.

The C subunit of the rat muscle enzyme is poorly characterized yet. Recently we developed a proce-

Enzyme investigated: Protein kinase (ATP: protein phosphotransferase), EC 2.7.1.37.

Abbreviations: ATP, adenosine 5′-triphosphate; cyclic AMP, adenosine cyclic 3′,5′-monophosphate; PK, protein kinase; cA-PK, cyclic AMP-dependent protein kinase; cA-PK II, cyclic AMP-dependent protein kinase isoenzyme II; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; BSA, bovine serum albumin; DEAE-cellulose, O-(diethylaminoethyl)-cellulose; CM-cellulose, O-(carboxymethyl)-cellulose; MOPS, N-2′-hydroxyethylpiperazine-2-ethanesulfonic acid; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; MES, 2-(N-morpholino) ethanesulfonic acid; ADA, N-(2-acetamido)-iminodiacetic acid; TRIS, tris(hydroxymethyl)-aminomethane.

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0341-0382/79/1200-1186 $ 01.00/0

dure for isolation of the enzyme in a single step, and preliminary characterized a few of its properties [7]. In the present publication we describe some further properties of the rat muscle enzyme and factors influencing its activity. Some of these factors, to which little or no attention has been paid so far in none of the characterization work carried out with C subunits of different sources, are of essential importance for the enzyme activity both *in vitro* and *in vivo*.

Materials and Methods

Histone (calf thymus, type II-A) was purchased from Sigma, protamine (salmon), cyclic AMP, reagents for polyacrylamide gels and zwitterionic buffers (MOPS, TES, HEPES, MES, ADA, TRIS) were obtained from Serva, Heidelberg; molecular weight standard proteins for SDS polyacrylamide gel electrophoresis were from BDH Chemicals, Poole (GB). Ampholine was from LKB, [γ^{32}-P]ATP (spec. act. > 20 Ci/mmol) from New England Nuclear. All materials were of highest grade available.

All operations were performed at 0–4 °C unless otherwise stated.

Purification of C subunit of cA-protein kinase II from rat muscle was achieved essentially by an improved form of the "single step" procedure [7] as described in detail elsewhere [37]. In principle, following chromatography of holoenzyme on DEAE cellulose and removal of type I enzyme(s) from the column by 0.1 M potassium phosphate buffer, the C subunit of isoenzyme II was released by cyclic AMP, eluted, and cochromatographed on CM-cellulose. Finally, the enzyme was dialyzed against 5 mM phosphate buffer and stored at 4 °C after addition of bovine serum albumin (0.1% w/v) which was found to have a stabilizing effect on the enzyme.

The standard assay of protein kinase activity was essentially as described elsewhere [8], one unit being defined as that amount of enzyme which transfers 1 pmol phosphoryl group from [γ-^{32}P]ATP to recovered protein during 5 minutes at 30 °C. The buffer system (MOPS) was adjusted to pH 6.8 or pH 7.8 for substrate histone and protamine, respectively. It was ensured that, at the final concentration of 50 mM, the buffer stabilizes these pH values during the entire length of the assay. If not otherwise stated, counterion of Mg^{2+} was acetate and sulfate in assays with histone and protamine, respectively. If necessary, enzyme preparations were diluted with an aquaous solution of bovine serum albumin (0.1% w/v) in order to get linear relations. Heat-denaturated enzyme preparation (60 °C, 10–15 min) served as negativ control in every set of determination. Further details are given in the text.

SDS gel electrophoresis and autoradiography of dried gels were essentially as described elsewhere [9]. Determination of protein was carried out essentially according to Lowry *et al.* [10] with bovine serum albumin as the standard. Radioactivity was measured in a Packard liquid scintillation counter Tricarb 3380. The ionic strength was determined conductometrically using a WTW-LF 42 apparatus. Ultraviolet light spectra were recorded in a Beckmann Acta M V. Isoelectric focusing and protein kinase assay in polyacrylamide gels were done as described in detail elsewhere [8]. The heat-stable inhibitor protein specifically acting on cA-protein kinase activities was isolated from rat muscle tissue according to the procedure given by Walsh *et al.* [11] with slight modifications.

Results and Discussion

The enzyme preparation revealed homogeneity on polyacrylamide gel electrophoresis in presence of SDS. It migrated in two bands, a major band slightly above ovalbumin and a minor band at the level of ovalbumin. This is identical to what we obtained previously [7] when the enzyme was prepared by the original "single step procedure" and gives a hint to a *molecular mass* in the range of 40–45 k. Similar results were published for the C subunit from rat pancreas [12]. Gel filtration on Sephadex (G 200 superfine) led to a molecular weight assessment of about 37 k (see [7]).

The *isoelectric point* was found at pH 9.0 ± 0.2 (Fig. 1) which appears to be somewhat higher than found previously [7] and reported for most of the C subunits from different tissues studied so far [37]. The enzyme focused as a single band, whereas our previous preparations obtained without the newly added concentration step on CM-cellulose showed two peaks [7]. Also, most of the C subunit preparations from different tissues were reported to migrate in more than one peak. On the other hand, treatment of holoenzyme with cyclic AMP before focusing generated a single C subunit peak moving to exactly the same pH as above [37]. This may eventually indicate that microheterogeneities found with C sub-

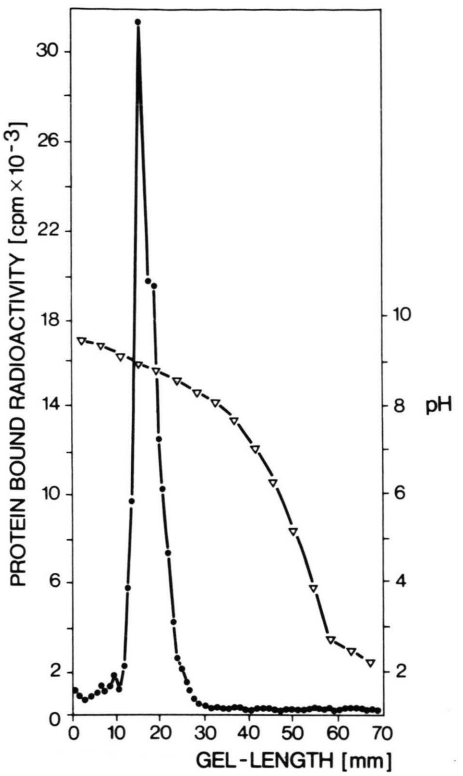

Fig. 1. Isoelectric focusing of C subunit of cA-protein kinase II from rat muscle followed by an enzyme assay in polyacrylamide gel. Enzyme preparation (2.2 µg protein) was applied in glycerol-ampholine mixture onto a 8 cm gel. Focusing was carried out at 4 °C for 12 h applying 100 V permanently. After removal of gel from the focusing device and equilibration to pH 7.8, the enzyme was assayed at 30 °C for 30 min within the complete gel using protamine as the substrate. This was followed by extraction of unspecific radioactivity, slicing of gel into 1.1 mm pieces, drying, and counting. For pH determination one extra gel was cut into 5 mm slices, minced, and suspended in distilled water.

unit preparations from various sources, may originate in the preparation conditions employed.

The *ultraviolet absorption* showed a maximum at 276 nm and a minimum at 250 nm; there was no indication of a chromophore group absorbing in the visible light range. This is in close correlation with the UV-spectrum found for the C subunit of rabbit skeletal muscle [6].

The *temperature stability* of the C subunit is demonstrated in Fig. 2. Temperatures up to 40 °C relatively weakly influenced the enzyme if employed up to 20 min (Fig. 2A). The activity remained on a level of 85–90% of control except at 40 °C, where activity decreased somewhat stronger on incubation longer than 5 min. At 50 °C a quite rapid loss in activity occured, less than 1% of the original activity remaining after 7 min. The half life of the enzyme at this temperature was slightly less than 1 min. This basically agrees with data reported for cA-protein kinases from other tissues [4, 13]. Only 1 min sufficed to reduce the activity to values below 0.5% and 0.1% at 60 °C and 80 °C or 100 °C (the latter two not shown), respectively.

To test whether or not a substrate stabilization of the enzyme exists, a phenomenon well-known for many enzymes under their respective physiological environments (*e. g.* [14]), C subunit was heated in presence of substrate histone or of cosubstrate ATP (Fig. 2B). Because activity was influenced weakly at 40 °C whereas at 50 °C a rapid loss occured, these two temperatures were chosen. Substrate histone did not show any stabilization at all. If anything, it seemed rather to destabilize the enzyme at both temperatures. (The phosphoryl group accepting capacity of histone itself was not influenced by these temperatures.) In presence of ATP but *absence* of Mg^{2+}, stability of C subunit clearly was lowered. At 40 °C the activity droped to 30% and 20% after 5 and 10 min, respectively, whereas 85% and 75% were left in the controls. At 50 °C enzyme inactivation was accelerated by ATP and the half life reduced to approximately 0.5 min. (ATP itself did not change its ability to serve as donor of phosphoryl groups.) In contrast, however, ATP in *presence* of Mg^{2+} did show a stabilizing effect. At 40 °C approximately 90% of activity remained after 10 min and at 50 °C still about 60% and 45% after 5 and 10 min, respectively. (Mg-ATP itself showed unchanged properties as cosubstrate after treatment at 50 °C.) Although different protein kinases (holoenzymes) were found to differ in this respect, our observation would seem to be in line with that of others. The temperature stability of a "kinase I" from red blood cells could be enhanced in presence of both ATP and Mg^{2+} [15].

Attempts to freeze the preparations have met with mixed success. It, nevertheless, is obvious that changes of temperature to beneath the freezing point markedly destroy enzymatic activity. For instance, one or two cycles of freezing (at −21 °C) and thawing (at 30 °C) reduced the activity to about 80% and 4 cycles to around 60%. However, the degree of inactivation varied and seemed to depend on the age of the enzyme preparation.

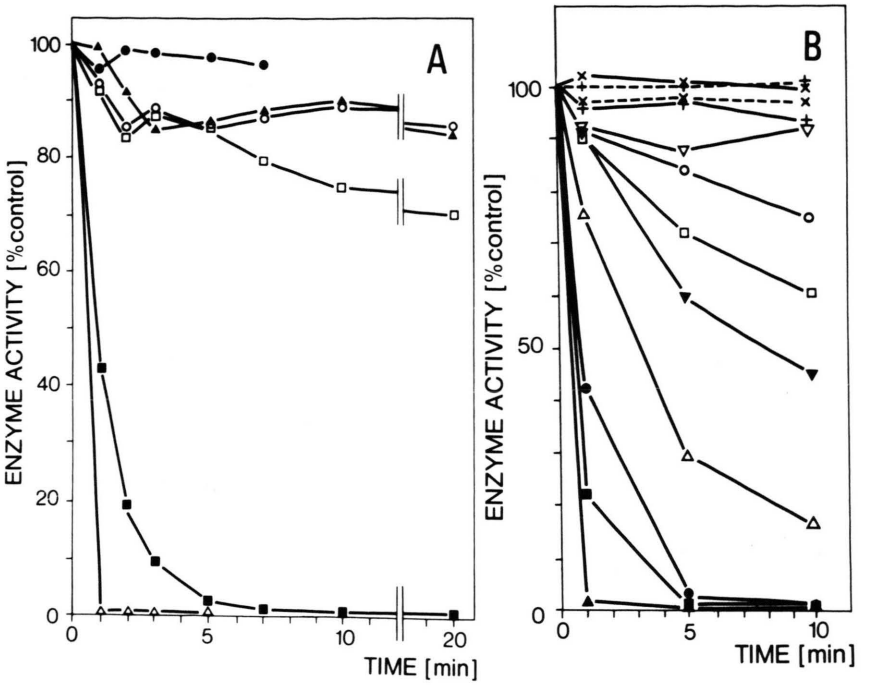

Fig. 2. Temperature stability of C subunit of cA-protein kinase II from rat muscle in absence (A), and in presence (B) of substrate histone or cosubstrate ATP. C subunit preparations diluted properly with a 0.1% (w/v) solution of bovine serum albumin were incubated in siliconized glass tubes: (A) at 26 °C (●), 30 °C (○), 35 °C (▲), 40 °C (□), 50 °C (■), or 60 °C (△) in a water bath under gentle shaking. Samples were withdrawn after 0 (control), 1, 2, 3, 5, 7, and 20 min and placed on ice (Eppendorf reaction tubes). (B) at 40 °C either alone (○) or in presence of histone (□), of ATP in *absence* of Mg^{2+} (△), or of ATP in *presence* of Mg^{2+} (▽). Ratios of mixtures as well as concentration of Mg^{2+} were as in the standard assay. This test was carried out identically at 50 °C: enzyme alone (●), mixtures of enzyme and histone (■), of enzyme and ATP in *absence* (▲) or *presence* (▼) of Mg^{2+}. Samples were withdrawn after 0 (control), 1, 5, and 10 min and placed on ice (Eppendorf reaction tubes). Kinase activity was assayed in triplicate according to the standard procedure using histone as substrate. Activities found were related to the respective control. To test for the stability of substrate and cosubstrate, histone in absence (−×−) and presence (- - - × - - -) of 0.1% bovine serum albumin, and ATP in absence (−+−) and presence (- - - + - - -) of 10 mM Mg^{2+} were incubated at 50 °C and employed in standard assays with untreated enzyme.

The *influence of protons* was studied in two different respects. First, to find out the optimum pH of enzyme activity and second, to test whether the depression of enzyme activity at both sides of the optimum is reversible or not.

The pH optima were found at about pH 6.8 and 7.8 for substrate histone and protamine, respectively (Fig. 3). However, these optima are relatively broad and ≥ 95% of maximal activity was found at pH 6.5 to 7.1 in case of histone, and at pH 7.3−8.1 in case of protamine. As far as determined, the optima of some other tissues and species are similar (*e. g.* [16−19]). Values of pH higher than 9 where not investigated since it is known that protamine as well as histone aggregate heavily under these conditions [20].

To test on the reversibility of the effect of high proton concentrations, the enzyme was dialyzed in presence of substrate histone against acetate or citrate buffer adjusted to different values between pH 2.2 and 6.2, and kept at the respective pH for about 18 h in the cold. As shown in Fig. 4, after readjustment to pH 6.8 by dialyzing against an appropriate phosphate buffer, enzyme activity was lost irreversibly in samples which were exposed to pH values lower 5. To exclude the possibility of an artifact by a destruction of substrate histone, it was treated identically as the enzyme preparation and employed in assays with untreated enzyme. Essentially, no change in its ability to function as acceptor of phosphoryl groups occured. This result, which is supported by some data in the literature [13], seems

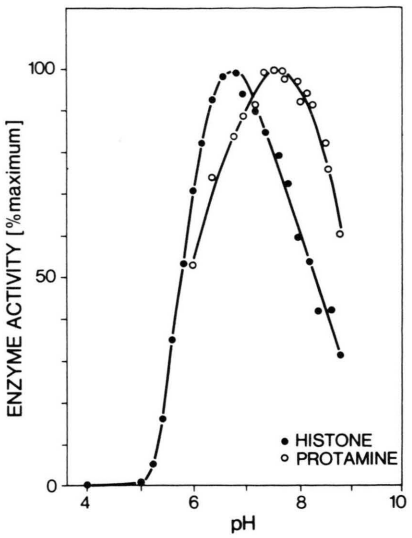

Fig. 3. Activity of C subunit of cA-protein kinase II from rat muscle as a function of pH. Buffer systems used were acetate (pH 4.0–6.2), MOPS (pH 6.0–8.2), and HEPES (pH 6.8–8.8) at a final concentration of 50 mM. All other components of the reaction mixture were as in the standard assay. Assay in presence of histone (●) or protamine (○).

worth to be emphasized, because of considerable practical implications. Some of the purification procedures of kinase involve an acid precipitation step [17] and it seems obvious that yields may heavily be affected. Further, for isoelectric focusing it shows that C subunit preparations must not be applied at the acidic side of the gel. It also explains the failure of attempts to separate the diverse kinase subunits of both isoenzymes on the basis of their different charges in a "single step"-like manner as we described for subunit C [7]. This had been tried at pH values below 5, since the holoenzyme at this pH was thought to be positively charged and could, hence, be bound to a cation exchanger; sububit R should then be released by cyclic AMP leaving subunit C on the resin.

The diminished enzyme activity on the basic side of the pH optimum seems, however, to be reversible. This is suggested by the isoelectric focusing experiments, for the enzyme is applied onto the gels at pH values around 9.5, focused for usually more than 12 h (in which time it moves to pH 9) and still shows activity in the subsequent kinase assay. The activity measured is, moreover, positively correlated with the enzyme units applied [8].

All protein kinases studied so far exhibit an absolute requirement for *divalent metal ions*, and Mg^{2+} is usually pointed out as the most effective [17, 18]. Table I summarizes the ability of diverse divalent metal ions to fulfil this requirement for the rat muscle enzyme. The most effective were Mg^{2+} and Co^{2+}; a partial replacement of these by Mn^{2+} was observed; all the others were ineffective. This is in line with results for other cA-protein kinases [17, 18] and even cyclic GMP-dependent protein kinases [21]. The optimum concentration for Mg^{2+} was found to be 5–10 mM (Fig. 5) but ≥ 90% of activity was elicited at 3–16 mM. The optimum of Co^{2+} was sharper and found at 0.5–0.8 mM; ≥ 90% of activity was expressed at 0.4–1.5 mM. At the respective optima, enzyme activity in presence of Co^{2+} was by a factor of 1.5 higher than in presence

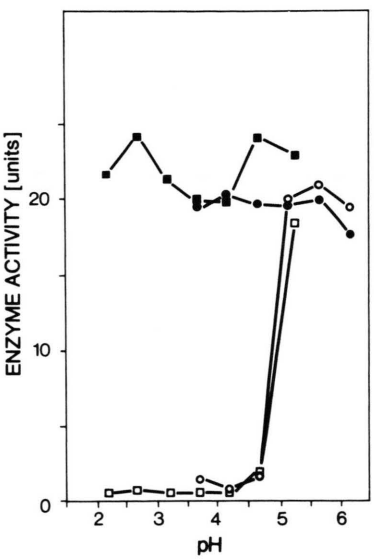

Fig. 4. Effect of exposure of C subunit of cA-protein kinase II from rat muscle to proton concentrations below pH 6.5. Properly diluted enzyme preparation was mixed with histone solution in a ratio as in the standard kinase assay. Samples therefrom or of the histone solution alone were dialyzed against 20 mM acetate buffer (enzyme-histone mixture ○; histone ●) adjusted to pH 3.7, 4.2, 4.7, 5.2, 5.7, or 6.2, or dialyzed against 20 mM citrate buffer (enzyme-histone mixture □; histone ■) adjusted to pH 2.2, 2.7, 3.2, 3.7, 4.2, 4.7, or 5.3 for about 18 h at 4 °C. Subsequent readjustment of samples to the original state was carried out by dialyzing against phosphate buffer pH 6.8, with 20 mM buffer solution for about the first 6 h followed by 5 mM buffer solution for about 15 h. Enzyme activities were determined in triplicate by the standard kinase assay using either treated enzyme-histone mixtures without further addition of histone, or treated histone and untreated enzyme.

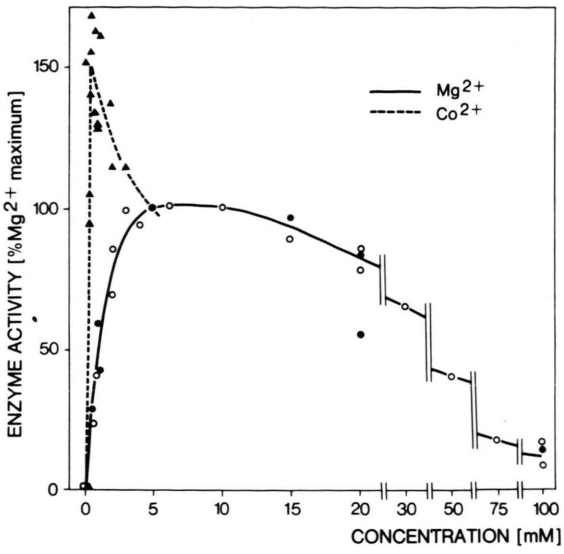

Fig. 5. Effect of Mg^{2+} and Co^{2+} on the activity of C subunit of cA-protein kinase II from rat muscle. Appropriate amounts of Mg-acetate (○) or Mg-chloride (●), or of Co-chloride (▲) were added to the reaction mixture to adjust the final salt concentration shown in the diagram. Enzyme activity was determined according to the standard assay with histone as substrate.

Table I. Effect of divalent metal ions on the activity of C subunit of cA-PK II from rat muscle. Enzyme activity was determined in triplicate as described in the standard assay with histone as substrate, except for the variation in the kind and concentration of the divalent metal ions as indicated. Absolute values were related to that obtained in presence of optimal concentration of Mg^{2+} (5 mM). Metals added carried identical counter ions as Mg^{2+} (acetate or chloride).

Metal ion		Enzyme activity [% Mg^{2+}-optimum]
Symbol	Final Concentration [mM]	
Mg^{2+}	5	100
	1	55
Co^{2+}	5	100
	1	140
Mn^{2+}	5	12
	1	26
Cu^{2+}	5	1.1
	1	0.6
Ca^{2+}	5	<0.5
	1	<0.5
Hg^{2+}	5	<0.5
	1	<0.5
no added metal		<0.5

of Mg^{2+}. Half-maximal activity was reached at 0.9 mM and 0.25 mM for Mg^{2+} and Co^{2+}, respectively.

If present simultaneously, the effect of Mg^{2+} and Co^{2+} on enzyme activity was found to be complex and could not be appointed to a certain type of interaction. Ca^{2+} antagonized Mg^{2+} in a dose-related manner. To get a more detailed information, reaction rate was measured at different concentrations of Mg^{2+} (1–10 mM) in presence of a certain concentration of Ca^{2+} (2, 1, or 0.5 mM). These measurements revealed a competitive-like interaction between Mg^{2+} and Ca^{2+}. However, double reciprocal plots did not show straight lines.

In course of examining the influence of the diverse compounds of our assay system on enzyme activity we found different *anions and zwitterions* to influence the enzyme activity unexpectedly diverse. Anions reach the assay mixture first of all as the counter ions of the metal added and the buffer system used. As is shown in Fig. 6, depending on the substrate employed, a dramatic influence is exerted by acetate and sulfate. In presence of acetate, a maximum of histone phosphorylation was observed, whereas with sulfate only 25–30% of the acetate value was reached. On the other hand, protamine phosphorylation was influenced inversely. In presence of sulfate, enzyme activity was about two times as high as in presence of acetate. These effects occured comparably in presence of both MOPS and acetate buffer. If phosphate buffer was used, these differential effects were practically lost. However, the use of phosphate buffer resulted in extremely small absolute values as compared with MOPS (see below). Chloride reduced both histone and protamine phosphorylation, the latter much more pronounced, whereas nitrate seemed to lower protamine phosphorylation only.

With different anions and zwitterions of diverse buffer systems, roughly the following order of enzyme activity was found: MOPS (100%) ≥ TES (95%) ≅ HEPES > MES (65%) ≅ acetate ⩾ADA (20%) > phosphate (15%) > TRIS · maleate (7%). Hence, systems like acetate or phosphate, which are frequently used in kinase assays, should better be avoided.

The enzymatic activity was influenced in two different aspects by the *ionic strength.* On the one hand, stability of enzyme preparations seemed to improve with increasing ionic strength of the final

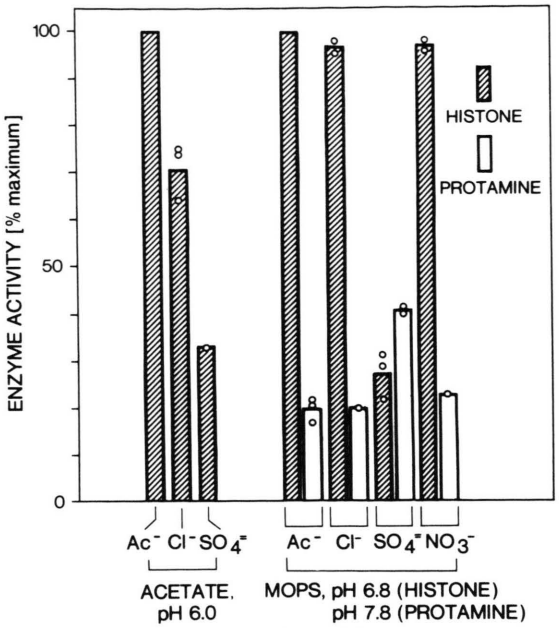

Fig. 6. Effect of anions on the activity of C subunit of cA-protein kinase II from rat muscle. Except for the variation in the kind of anions, enzyme activity was determined according to the standard assay with histone (hatched columns) or with protamine as the substrate. Final concentration of the respective anions was 10 mM; anions were added to the reaction mixture as the counter ion of Mg^{2+}.

buffer. This agrees with data in the literature (*e. g.* [18, 22]). On the other hand, increase of ionic strength in enzyme assays by increasing the concentration of salt or of buffer resulted in diminished enzyme activity. For instance, increasing the final concentration of buffer (acetate buffer, pH 6.0) from 50 mM to 100 mM in enzyme assays decreased kinase activity by nearly 60%.

The rat muscle enzyme is sensitive to a *heat-stable inhibitor protein* isolated from rat muscle. Heat-stable proteins of this kind are known to specifically affect C subunits of cA-protein kinases; lack of tissue and species specificity permitting a general applicability as a criterium for cA-protein kinase activities [23, 24]. In a typical experiment, histone phosphorylation by 117 units of C subunit (0.6 µg protein) was reduced to 50% by 0.4 µg of inhibitory protein. In comparison, inhibition of protamine phosphorylation was less pronounced. It was ensured that the inhibitor preparation does not possess any ATPase or phosphoprotein phosphatase activity. This, therefore, evidences that the enzyme used is in fact cA-protein kinase derived.

The rat muscle enzyme has been demonstrated to phosphorylate a variety of serum proteins as well as of surface proteins of mammalian cells [9, 25–27]; histones of calf thymus or rat liver and salmon sperm protamine were found to be excellent substrates. In histones, only seryl residues are phosphorylated but seryl as well as threonyl in cell surface proteins. For the more detailed characterization of the kinase reaction described in this paper, we used histone and protamine. These are suitable substrates for several reasons. Both are well-known substrates *in vivo* where they seem to be mutually exclusive like in spermatogenesis in particular (reviewed [1]), they are frequently employed in *in vitro* studies, are likely to be randomly coiled in aqueous solution at around neutral pH [28], and, although some internal homologies exist between them [29], they may be classified differently: Histones clearly belong to proteins whereas protamines are rather polypeptides or, better, polyamino acids because of their low molecular weights and extremely high arginine content.

The *initial velocities* of the phosphorylation reaction at varying concentrations of cosubstrate ATP or of substrates histone and protamine are presented in Fig. 7. To find out whether classical Michaelis-Menten kinetic is followed, and to estimate Michaelis-constant (K_M) as well as maximal velocity (V), the curvilinear plots (insets in Fig. 7) of initial velocities (v) against concentrations (c) of ATP (c_{ATP}) or of substrate protein (c_{SUBSTR}) were linearly transformed according to $v = V + K_M(v/c)$ (Fig. 7) and to $(1/v) = (1/V) + (K_M/V) \cdot (1/c)$ (not shown). Because neither v nor c are errorless, these transformations are not mere variants of $v = (V \cdot c/K_M + c)$ but may lead to an estimation of K_M and V with different accuracy in case unweighted data are used [30]. That both forms of transformation resulted in good fitting straight lines evidences that the reactions fulfil in fact the Michaelis-Menten equation with good agreement. Or, in other words, it shows that the concentration of the intermediary complexes of enzyme/cosubstrate/substrate seems to be relatively constant during the period of time the enzyme action is allowed to take place. For ATP, the apparent K_M is nearly identical in presence of both substrate proteins and may be estimated with 3 to 4 µM (Fig. 7A). The exact values found were 3.4 µM and 3.8 µM in presence of histone and protamine, respectively. Values given in the literature for different tissues and species are similar [4, 18, 31], but

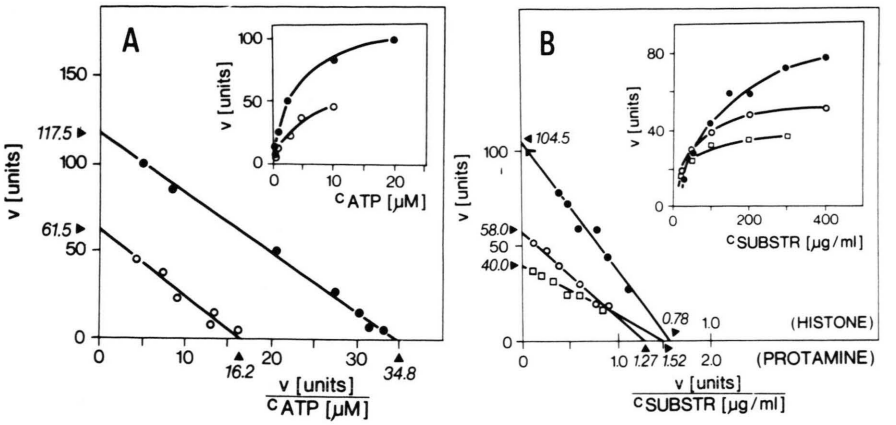

Fig. 7. Initial velocities of phosphorylation reaction catalyzed by C subunit of cA-protein kinase II from rat muscle at varying concentrations of (A) cosubstrate ATP, and (B) substrate histone or protamine. Enzyme assays were carried out according to the standard procedure except for the variation in concentration of: (A) ATP in presence of histone at pH 6.8 (●) or of protamine at pH 7.8 (○). (B) histone at pH 6.8 (●) and protamine at pH 6.8 (□) or 7.8 (○) at a fixed concentration of ATP (40 μM). Initial velocities (v) at varying concentrations of ATP (c_{ATP}) or of protein substrates (c_{SUBSTR}) obeying Michaelis-Menten equation, were transformed according to $v = V + K_M (v/c)$.

usually in a range of 10–20 μM [6, 15, 17, 19, 22]. Values of V varied among preparations. For the substrate proteins, the apparent K_M values may be estimated with 135 μg/ml and 46 μg/ml for histone and protamine, respectively (Fig. 7 B). If the pH was adjusted to pH 6.8 (optimum for histone phosphorylation), the K_M for protamine seemed to decrease. As an extreme, 26 μg/ml were measured. Hence, application of the optimum for histone phosphorylation to other proteins, as done by some groups (*e. g.* [4]), may be misleading. Analysis of substrates and products by electrophoresis on polyacrylamide gels in presence of SDS and subsequent autoradiography revealed that phosphorylation of histones occured preferentially in fractions H2B and H2A, which is supported by data of others [31]. Because the molecular weights of both histone fractions are in the range of 14 k [32], the apparent K_M value for histone may roughly be estimated with 9–10 μM. Protamines constituting salmine are known to be less heterogeneous than histones in both size and composition, their molecular weights being in the range of 4–5 k [29]. Using this molecular weight, the apparent K_M value for protamine may also be approximated with about 10 μM (5–6 μM at pH 6.8). These K_M values are of the same order of magnitude as found for other tissues and species [13, 15, 17, 31, 33, 34] or clearly smaller [6, 35, 36]. However, at very low protein concentrations ($< K_M$), values tended to deviate, the plots becoming multiphasic. This is similar to observations by others [17]. It may be explained by processes like adsorption at the glass wall of reaction tubes (which is known to affect highly diluted protein solutions) or by changes in the aggregation of substrate molecules. The latter is an extremely complex event since selfaggregation within one fraction of histones as well as co-aggregation with other fractions is known; aggregation seems to increase with increasing protein concentration [20]. It seems obvious that larger changes of aggregation patterns may alter kinetic data because of appearance or disappearance of phosphorylation sites.

The maximal velocity of the phosphorylation reaction is considerable higher with histone than with protamine, the relative ratio being about 100 : 40 when measured at the respective optimal conditions. It therefore appears as if these substrates may possess comparable affinities to the C subunit but that protamine has a higher "stickiness" to it decreasing its turnover to beneath that of histone. Utilization of histone in preference to protamine as with the rat muscle enzyme has been found for cA-protein kinases derived from various tissues and species ([4, 31] and references therein). The ratio found, however, varies widely with parameters like the buffer system used or the kind of anions present in the assay. As demonstrated, certain conditions may even lead to a just inverted ratio. These indicate that the conditions chosen for the phosphorylation of a partic-

ular protein may in part explain "substrate specificity" of a certain protein kinase resulting in definitions like "hisone-kinase", "protamine-kinase", and the like.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft.

[1] R. A. Jungmann and D. H. Russel, Life Sci. **20,** 1787 – 1798 (1977).
[2] D. A. Walsh and E. G. Krebs, The Enzymes, **Vol. 8,** (P. D. Boyer, ed.), pp. 555 – 581, Acad. Press, New York 1973.
[3] H. G. Nimmo and P. Cohen, Adv. Cyclic Nucleotide Res. **8,** 145 – 266 (1977).
[4] H. Yamamura, K. Nishiyama, R. Shimomura, and Y. Nishizuka, Biochemistry **12,** 856 – 862 (1973).
[5] J. D. Corbin, S. L. Keely, and C. R. Park, J. Biol. Chem. **250,** 218–225 (1975).
[6] P. J. Bechtel, J. A. Beavo, and E. G. Krebs, J. Biol. Chem. **252,** 2691–2697 (1977).
[7] V. Kinzel and D. Kübler, Biochem. Biophys. Res. Commun. **71,** 257–264 (1976).
[8] M. Gagelmann, W. Pyerin, D. Kübler, and V. Kinzel, Anal. Biochem. **93,** 52–59 (1979).
[9] V. Kinzel and D. Kübler, Exp. Cell Res. **113,** 173–182 (1978).
[10] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[11] D. A. Walsh, C. D. Ashby, C. Gonzalez, D. Calkins, E. H. Fischer, and E. G. Krebs, J. Biol. Chem. **246,** 1977–1985 (1971).
[12] G. Marchis-Mouren, P. Mangeat, and H. Chahinian, Phosphorylated Proteins and Related Enzymes, Amino-acid, Peptide and Protein Abstracts, Special Publication **1** (E. S. Krudy and P. Clare, eds.), pp. 33–43, Information Retrieval, London/Washington DC 1977.
[13] I. Uno, T. Ueda, and P. Greengard, J. Biol. Chem. **252,** 5164–5174 (1977).
[14] F. J. Wiebel and H. V. Gelboin, Chemical Carcinogenesis Essays, (R. Montesano and L. Tomatis, eds.), pp. 57–80, IARC Sci. Publ. **10,** Lyon 1974.
[15] M. Tao, Meth. Enzymol. **38,** 315–322 (1974).
[16] B. Jergil and G. H. Dixon, J. Biol. Chem. **245,** 425–434 (1970).
[17] E. Miyamoto, J. F. Kuo, and P. Greengard, J. Biol. Chem. **244,** 6395–6402 (1969).
[18] P. H. Sugden, L. A. Holladay, E. M. Reimann, and J. D. Corbin, Biochem. J. **159,** 409–422 (1976).
[19] L.-J. Chen and D. A. Walsh, Meth. Enzymol. **38,** 323–329 (1974).
[20] E. W. Johns, Histones and Nucleohistones, (D. M. P. Phillips, ed.), pp. 1–45, Plenum Press, London/New York 1971.
[21] M. Shoji, J. G. Patrick, C. W. Davis, and J. F. Kuo, Biochem. J. **161,** 213–221 (1977).
[22] C. S. Rubin, J. Erlichman, and O. M. Rosen, Meth. Enzymol. **38,** 308–315 (1974).
[23] J. A. Traugh, C. D. Ashby, and D. A. Walsh, Meth. Enzymol. **38,** 290–299 (1974).
[24] E. G. Krebs, Curr. Top. Cell Regul. **5,** 99–133 (1972).
[25] V. Kinzel and G. C. Mueller, Biochim. Biophys. Acta **322,** 337–351 (1973).
[26] V. Kinzel, A. Alonso, and D. Kübler, Eur. J. Biochem. **55,** 361–368 (1975).
[27] V. Kinzel, D. Kübler, A. M. Mastro, and E. Rozengurt, Biochim. Biophys. Acta **434,** 281–285 (1976).
[28] C. Tanford, Adv. Protein Chem. **23,** 121–282 (1968).
[29] D. M. P. Phillips, Histones and Nucleohistones, (D. M. P. Phillips, ed.), pp. 47–83, Plenum Press, London/New York 1971.
[30] J. E. Dowd and D. S. Riggs, J. Biol. Chem. **240,** 863–869 (1965).
[31] J. F. Kuo and P. Greengard, Biochim. Biophys. Acta **212,** 434–440 (1970).
[32] E. M. Bradbury and C. Crane-Robinson, Histones and Nucleohistones, (D. M. P. Phillips, ed.), pp. 85–134, Plenum Press, London/New York 1971.
[33] J. F. Kuo, B. K. Krueger, J. R. Sanes, and P. Greengard, Biochim. Biophys. Acta **212,** 79–91 (1970).
[34] B. E. Kemp, D. J. Graves, E. Benjamini, and E. G. Krebs, J. Biol. Chem. **252,** 4888–4894 (1977).
[35] M. A. Brostrom, E. M. Reimann, D. A. Walsh, and E. G. Krebs, Adv. Enzyme Regul. **8,** 191–203 (1970).
[36] D. B. Bylund and E. G. Krebs, J. Biol. Chem. **250,** 6355–6361 (1975).
[37] D. Kübler, M. Gagelmann, W. Pyerin, and V. Kinzel, Hoppe-Seyler's Z. Physiol. Chem. **360,** 1421–1431 (1979).