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DISTINCT CONFORMATIONAL CHANGES IN THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE AROUND PHYSIOLOGICAL CONDITIONS. DO THESE CHANGES REFLECT AN ABILITY TO ASSUME DIFFERENT SPECIFICITIES?

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SUMMARY

The free catalytic subunit of cAMP-dependent protein kinase readily undergoes a pronounced, salt-induced conformational change at neutral pH and around physiological values of ionic strength. This change, which is fully reversible, can be monitored directly by the relative chemical reactivity of two SH groups in the enzyme. Upon increasing the ionic strength of the medium from 0.03 to 0.22, one sulfhydryl becomes more reactive towards 5,5'-dithiobis[2-nitrobenzoic acid] while the other sulfhydryl becomes less reactive towards the same reagent. In parallel, the enzyme undergoes a salt-induced inactivation when histone H2b is used as a substrate. Though not reflected in the V_{max}, this conformational change considerably increases the K_m of the enzyme for histone H2b as well as for MgATP. This intrinsic malleability of the enzyme can account for the well-known salt inhibition of the enzyme for certain substrates and ion-dependent activation towards other substrates. It is suggested that this malleability might constitute the molecular basis for modulating the specificity of the enzyme and channeling its activity from one substrate to another in response to intracellular specifier signals.

It is well established now that enzymes often undergo distinct conformational changes upon binding their biological ligands (substrates, coenzymes, inhibitors, activators, etc.). These conformational changes play an important role in forming the catalytically active structure (by an "induced fit" [1]), in providing the means for feedback regulation (e.g. through "allostery" [2]) and in diverting the action of a given enzyme towards a different substrate (by means of a "specifier protein" [3]).

In contrast to enzymes that display a sharp specificity, cAMP-dependent protein kinase (cAMPdPK) is known to phosphorylate a wide variety of proteins at least in vitro and probably even in intact cells [4]. It becomes therefore imperative to elu-

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Abbreviations: C, the catalytic subunit of cAMP-dependent protein kinase; cAMP, adenoside 3':5'-monophosphate; cAMPdPK, cAMP-dependent protein kinase; Nbs_2, 5,5'-dithiobis[2-nitrobenzoic acid].

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cidate the mechanism by which the kinase activity may be channeled *in vivo* to bring about a specific phosphorylation at a given time and a given locus within the cell, in response to a distinct metabolic signal. This paper addresses itself to this question and presents evidence showing that the free catalytic subunit of cAMPdPK has a malleable structure, a property which may reflect a mechanism for diverting the affinity (and thus the action) of this enzyme from one target substrate to another.

MATERIALS AND METHODS

Proteins and Chemicals. The free catalytic subunit of cAMPdPK was purified by the procedure of Beavo *et al.* [5]. Histone H2b was prepared by the method of Böhm *et al.* [6]. [γ-^{32}P] ATP (20-27 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. All other chemicals were the best available grade from commercial sources.

Chemical Modification of C with Nbs_2. Freshly prepared enzyme solutions were preincubated with dithioerythritol (3 mM) for 1 h at 37° prior to the chemical modification. The enzyme solution was then freed from low-molecular-weight thiol compounds by gel filtration on a column of Sephadex G-25 (fine), equilibrated and run (at 23±1°) with a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0. The modification of SH groups was monitored by determining the nitrothiobenzoate anion at 412 nm [17]. This modification was carried out under pseudo first-order conditions (Nbs_2 concentration >50-fold higher than the molar concentration of the enzyme). In the calculation of rate constants for the modification of SH groups, corrections were made to take into account the contribution of the slower reacting SH groups, according to Ray and Koshland [8]. Second-order rate constants were calculated from the linear dependency of the pseudo first-order rate constants on the concentration of Nbs_2.

Assay of cAMPdPK. The assay was based on the phosphorylation of histone H2b with [γ-^{32}P] ATP as described elsewhere [9]. For the determination of initial velocities (Table 1) the assay medium used contained 3-(M-morpholino)-propanesulfonic acid (20 mM) $MgCl_2$ (10 mM), ethyleneglycol bis (β-aminoethyl-ether) N,N'-tetraacetic acid (0.25 mM), 2-mercaptoethanol (0.25 mM), substrates and enzyme (at the indicated concentrations), pH 7.0. Assays were performed for 30 and 60 sec at 30°, and in all cases, linear incorporation of [^{32}P] into histone H2b was ascertained during the 60 sec assay. The ionic strength of the medium was adjusted with NaCl.

Protein Concentrations. Protein concentrations were determined by the method of Lowry *et al.* [10] with bovine serum albumin as a reference standard.

RESULTS AND DISCUSSION

Classifying the SH Groups of C by their Chemical Reactivity towards Nbs_2. When the SH-groups of C are allowed to react with an excess of Nbs_2 (at neutral pH and low ionic strength) and the extent of modification is followed with time, the titration curve levels off at about 2.3 moles of SH groups per mole of C (Fig. 1A). Beyond that point no additional titrable sulfhydryls could be detected even upon inclusion of sodium dodecylsulfate (final concentration 2%) in the reaction mixture (data not shown). Upon plotting the time course of the reaction as pseudo first-order kinetics (Fig. 1B) two clear-cut phases can be distinguished: a slower phase with a rate constant k_1 = $(2\pm0.3)\times10^2 M^{-1}min^{-1}$, corresponding to the titration of 0.9±0.1 moles of SH groups per mole of C, which will be referred to as SH_I, and a faster phase with a rate constant

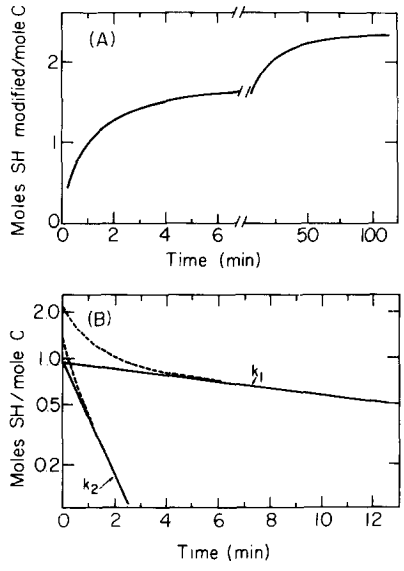

FIGURE 1: Rate of reaction of the sulfhydryl groups in C with Nbs_2. The reaction mixture (1.05 ml) contained pure C (4.8 µM) in a buffer composed of Hepes (0.1M) and EDTA (1 mM), pH 7.0. The reaction was initiated by addition of 50 µl of a solution of Nbs_2 (5 mM, in the same buffer) to 1 ml of the enzyme solution and allowed to proceed at 23±1°C. Panel A: Time course of the modification of SH groups monitored spectrophotometrically (at 412 nm) vs. a reference cell containing a solution prepared identically but without enzyme. Panel B: First order plots for the modification of the SH groups in C by Nbs_2. The rate constants k_1 and k_2 are calculated from an analysis of the data presented in Panel A according to Ray and Koshland (8).

$k_2=(3.0\pm0.5)\times10^3 M^{-1}min^{-1}$, corresponding to the titration of 1.0±0.1 moles of SH groups per mole of C, which will be referred to as SH_{II}. Extrapolation to time zero of either one of the reaction traces (Fig. 2B) suggests the possible existence of an additional very fast phase, corresponding to 0.3±0.2 moles of SH groups per mole of C with a rate constant $k_3 > 10^4 M^{-1}min^{-1}$. In view of the fact that three cysteic acid residues per mole of C were determined after performic acid oxidation and amino acid analysis of the enzyme [9,11-14] it seems reasonable to attribute the above mentioned fast phase to the existence of an additional class of sulfhydryls (SH_{III}) which could be so reactive that it might be partially oxidized in the course of the purification of the enzyme, or during the manipulations carried out prior to the titration with Nbs_2.

The Sulfhydryls in C as Built-in Reporter Groups that Detect a Salt-Induced Conformational Change. A kinetic analysis of several experiments, in which the SH groups of C were titrated with Nbs_2 under identical conditions, except for the concentration of added NaCl (changing the ionic strength from 0.03 to 0.22) revealed that the rate constant k_1 and k_2 were considerably altered. Unexpectedly, the change in the rate constants occurred in opposing directions: while k_2 decreased from $3.4\times10^3 M^{-1}min^{-1}$

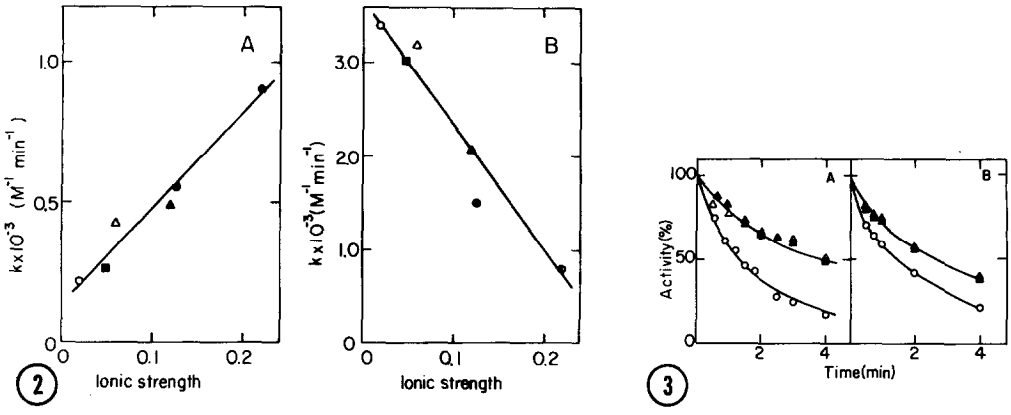

FIGURE 2: Effect of ionic strength on the rate constants k_1 and k_2. The reaction mixtures (all at pH 7.0) included: (a) C (4 µM), Hepes (0.1 M), EDTA (1 mM) (○); (b) same as (a) plus NaCl (0.1 M) (●); (c) same as (a) plus NaCl (0.2 M) (◐); (d) C (2.2 µM), Hepes (50 mM), EDTA (1 mM) and NaCl (30 mM) (■); (e) C (3.5 µM), Hepes (0.1 M), EDTA (1 mM) and $Mg(CH_3COO)_2$ (20 mM) (▲); (f) C (5.7 µM), potassium phosphate (50 mM), EDTA (1 mM) (▲). Panels (A) and (B) depict the effect of ionic strength on k_1 and k_2 respectively. In all cases the reaction was initiated by addition of a 50-fold molar excess of Nbs_2 over the enzyme concentration. The rate constants k_1 and k_2 were determined as described under Methods.

FIGURE 3. Effect of salt concentration on the rate of inactivation of C by Nbs_2 [panel A] and by 2,2'-dipyridyl disulfide [panel B]. [A]: The reaction mixtures (all at pH 7.0) included: (a) C subunit (4 µM), Hepes (0.1 M), EDTA (1 mM) (○); same as (a) plus NaCl (0.1 M) (▲); same as (a) plus NaCl (0.2 M) (△). The reaction was initiated in each case by addition of Nbs_2 (to a final concentration of 215 µM). [B]: The reaction mixtures included: (b) C subunit (4.8 µM), Hepes (0.1 M), EDTA (1 mM), pH 7.0 (○); same as (b) plus NaCl (0.1 M) (▲); same as (b) plus NaCl (0.2 M) (△). The reaction was initiated by addition of 2,2'-dipyridyl disulfide (to a final concentration of 190 µM). In order to follow the inactivation of the enzyme the reaction was stopped, for [A] and [B], at the indicated times by removing aliquots of 25 µl from the reaction mixtures and diluting them into 2.5 ml of an ice-cold buffer composed of 2-(N-morpholino)-ethanesulfonic acid (0.1 M), pH 6.5. A sample (50 µl, 50-100 ng) of this diluted enzyme was immediately assayed.

(at ionic strength 0.03) to $0.9 \times 10^3 M^{-1} min^{-1}$ (at ionic strength 0.22) i.e. by ∿3.8 fold k_1 increased from $2 \times 10^2 M^{-1} min^{-1}$ to $9 \times 10^2 M^{-1} min^{-1}$, i.e. by ∿4.5 fold (Fig. 2).

Now, the rate of chemical modification of low-molecular-weight sulfhydryl compounds (such as glutathion) is not significantly affected within the narrow range of ionic strengths used here [15]. Moreover, since the modification of the two SH classes is affected by the change in ionic strength in opposite directions, it seems plausible to assume that the change in the relative reactivity of the sulfhydryl classes is a reflection of a change in their microenvironment (steric availability, surrounding dielectric constant, dislocation of adjacent interacting groups, etc.). In other words, the enzyme seems to undergo a pronounced conformational change upon merely increasing the ionic strength of its medium from 0.03 to 0.22.

Under the conditions of the above experiment, Nbs_2 is negatively charged. Therefore the changes in k_1 and k_2 could, in principle, arise from an effect of ionic

strength on the adsorption of the reagent onto charged loci in the enzyme prior to the covalent reaction with the sulfhydryl groups, i.e. from an effect of salt on the ionic interactions between the reagent and the enzyme rather than on the conformation of the enzyme per se. In order to exclude this possibility, we compared Nbs_2 with its analog 2,2'-dipyridyl disulfide which has no net charge under the conditions of these experiments (pK_a of the pyridinium nitrogen of this analog was reported to be 2.45 [16]). This comparison was carried out by monitoring the inactivation of the enzyme by either one of the two reagents at different salt concentrations. As seen in Fig. 3, the inactivation of the enzyme by both the negatively charged Nbs_2 and its neutral analog, were found to be similarly affected upon changing the ionic strength, in agreement with our suggestion that the effect of ionic strenth on k_1 and k_2 arises indeed from a salt-induced structural change in the enzyme itself.

It should be noted that the experiments depicted in Fig. 2 were carried out with different ionic compositions, yet when the rate constants obtained were plotted vs. ionic strength they fell on one straight line, suggesting that the conformational change is ionic-strength-dependent rather than ion-specific.

Kinetic Analysis of the Salt-Induced Inactivation of C when Histone H2b is used as Substrate. The fact that phosphorylation of histones (or casein) by cAMPdPK is inhibited upon increasing the ionic strength of the assay medium was observed in several laboratories [17-19]. In principle, this could be due to an effect of ionic strength on these particular protein substrates, or on the enzyme itself, or on both. In fact some authors have suggested that this inactivation is primarily due to an effect of salt on the substrate [18]. Our finding that the enzyme itself undergoes a pronounced salt-induced conformational change around physiological values of ionic strength and pH, in the absence of any substrate whatsoever, makes it likely that the inactivation might be due (at least in part) to an effect of salt concentration on the enzyme.

In view of the above, we carried out a detailed kinetic analysis of the enzyme inhibition, in an attempt to find out which of the kinetic parameters of the enzyme (maximal velocity or Michaelis constants) may be responsible for the salt-induced inactivation of the enzyme.

The steady-state initial velocity (v) for a bisubstrate enzyme-catalyzed reaction following a linear pathway is given by the expression [20]:

$$v=VAB/(K_{AB}+AK_B+BK_A+AB)$$

where A and B stand for the concentrations of the substrates (in this case MgATP and histone H2b respectively), V for the maximal velocity, K_A and K_B for the Michaelis constants with respect to A and to B and K_{AB} is a constant defined by Alberty [21].

The maximal velocity (V) as well as the Michaelis constants K_A and K_B can be determined from the intercepts of the double reciprocal plots of the initial rate as

TABLE 1

Effect of NaCl Concentration on the Maximal Velocity and the Michaelis Constants of the C subunit of cAMPdPK[a]

Kinetic Constant	Final Concentration of Added NaCl (M)		
	-	0.03	0.11
K_A (for MgATP) (μM)	5 ± 1	15 ± 4	17 ± 3
K_B (for histone H2b) (μM)	8 ± 1	17 ± 5	32 ± 9
$V^{(b)}$ (nmoles [γ-^{32}P] incorporated/min)	1.2 ± 0.2	1.6 ± 0.3	1.6 ± 0.4

[a] In addition to the enzyme (in all cases between 50 and 70 ng) and the buffer described under Materials and Methods (μ=0.035) the assay mixtures contained in one series of experiments several constant concentrations of ATP (17.5; 9.4; 6.3; and 4.7 μM), each one with the following concentrations of histone H2b (μM): 34.8; 7.8; 5.8 and 3.4. In another series of experiments the concentration of histone H2b was kept constant (at each one of the values 34.8; 7.8; 5.8 and 3.4 μM) and the concentration of ATP (μM) varied (for each case) as follows: 17.5; 9.4; 6.3 and 4.7. Each series of experiments was carried out with the three indicated concentrations of NaCl. The kinetic parameters were calculated as described in the text.

[b] Maximal velocity.

a function of the concentration of one substrate (A or B) at varying concentrations of the other substrate (B or A, respectively). Furthermore, by carrying out the assay with varying NaCl concentrations in the assay medium, one can find out how each one of the kinetic parameters of the enzyme is affected by the ionic strength.

As seen in Table 1, upon increasing the concentration of NaCl in the assay medium, the catalytic activity of the enzyme assayed with histone H2b as substrate is inhibited due to a significant increase in the Michaelis constants of the enzyme for both histone H2b and MgATP, whereas the maximal velocity of the enzyme remains essentially unaffected. In other words, the inhibition of the enzyme seems to arise from a decrease in its affinity towards its substrates.

Interestingly, both Michaelis constants (K_A and K_B) increase similarly (by 3.4 to 4 fold) upon raising the NaCl concentration from 0 to 0.11 M, in spite of the fact that one of the substrates is a protein which, in principle, could undergo salt-induced structural changes (in its conformation or in its state of aggregation), while the other substrate, being a nucleotide, is not likely to undergo conformational changes under these conditions, so that the change in the Michaelis constant in this case is most probably due mainly to a conformational change in the enzyme itself.

Possible Physiological Implications. The basic finding reported in this communication is that the C subunit of cAMPdPK has a malleable structure and that it is capable of

undergoing pronounced conformational changes which can be monitored directly by the relative chemical reactivity of the SH groups of the enzyme. These conformational changes, which are demonstrated here to occur even in the absence of substrate, are fully reversible, take place at neutral pH upon slight modulation of ionic strength around physiological values, and bring about an inactivation of the enzyme toward a given substrate, in this case, histone H2b. The inactivation is shown to result from a decrease in the apparent affinity of the enzyme for this substrate without impairing the potential catalytic activity of the enzyme, measured by its maximal velocity.

Being an intrinsic property of the enzyme, the structural malleability of the C subunit might account (at least in part) for the well-known salt inhibition of the catalytic activity of this enzyme toward certain substrates [17-19], as well as for the fact that in the presence of certain ions (e.g. Mg^{++}) the enzyme may become activated toward other substrates [22]. At the same time, these observations may also account for the somewhat contradictory results obtained in different laboratories (depending on the specific reaction conditions used) with regard to the role played by the various SH groups in the catalytic activity of the enzyme [9,11-14].

Furthermore, the intrinsic malleability of C might constitute the molecular basis for the action of the various inhibitors [23-24] and modulators [25-26] of this enzyme, which apparently regulate the action of C even after it has been released from its "stored" form (undissociated cAMPdPK). It might be inferred from the foregoing that C is capable of assuming different conformations, with probably different specificities, and thus have its activity diverted from one substrate to another, where and when the need arises.

Reports from several laboratories (cf. [27]) indicate that the specific response of cells to hormones functioning via cAMP probably depends on factors other than the hormone receptors on the cell surface and the substrates available for protein kinase. The malleability of the C subunit, if designed to detect specifier signals in vivo, might provide a means for sharpening the specificity of the cellular response to hormones.

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