Toste, A. P., & Cooke, R. (1979) *Anal. Biochem. 95*, 317–328.
Van Derliyn, P., Barrio, J. R., & Leonard, N. J. (1978) *J. Biol. Chem. 253*, 8694–8696.
Webb, J. L. (1963) in *Enzyme and Metabolic Inhibitors*, Vol. 1, pp 507–512, Academic Press, New York.
Weber, B. H., Willeford, K., Moe, J. C., & Piszkiewicz, D. (1979) *Biochem. Biophys. Res. Commun. 86*, 252–258.
Wedler, F. C., & Gasser, F. J. (1974) *Arch. Biochem. Biophys. 163*, 57–78.
Wild, J. R., Foltermann, K. F., & O'Donovan, G. A. (1980) *Arch. Biochem. Biophys. 201*, 506–517.
Wilson, J. E. (1976) *Biochem. Biophys. Res. Commun. 72*, 816–824.
Winlund, C. C., & Chamberlin, M. J. (1970) *Biochem. Biophys. Res. Commun. 40*, 43–49.
Winlund-Gray, C., Chamberlin, M. J., & Gray, D. M. (1973) *J. Biol. Chem. 248*, 6071–6079.
Witt, J. J., & Roskoski, R., Jr. (1980) *Biochemistry 19*, 143–148.
Yon, R. (1972) *Biochem. J. 129*, 311–320.

Salt-Induced Conformational Changes in the Catalytic Subunit of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase. Use for Establishing a Connection between One Sulfhydryl Group and the γ-P Subsite in the ATP Site of This Subunit†

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ABSTRACT: The sulfhydryl groups in the catalytic subunit (C) of cAMP-dependent protein kinase are shown to behave as built-in reporter groups, whose relative chemical reactivity can be used to demonstrate that C readily undergoes a salt-induced conformational change at neutral pH and around physiological values of ionic strength. Upon increasing the ionic strength of the medium from 0.03 to 0.22, one SH group in C becomes more reactive toward 5,5′-dithiobis(2-nitrobenzoic acid) (the rate constant increases ~4.5-fold) while the other SH group in C becomes less reactive toward the same reagent (the rate constant decreases ~3.8-fold). Modification of the SH groups of C by this reagent brings about an inactivation of the enzyme which, at low ionic strength, can be shown to occur concomitantly and stoichiometrically with the modification of one (kinetically characterized) sulfhydryl. When ATP and its analogues are used to protect the enzyme from inactivation by this reagent, a connection is established between this SH group and the γ-P subsite of the ATP binding site in C. In parallel with the above-mentioned salt-induced conformational change, the C subunit undergoes an inactivation (which increases with ionic strength) as measured by histone H2b phosphorylation. Though not reflected in the V_{max}, this conformational change considerably increases the K_m of the enzyme for histone H2b (~4-fold) as well as for MgATP (~3.4-fold). This intrinsic malleability of the enzyme, shown here to occur even in the absence of substrate, can account for the well-known salt inhibition of the enzyme for certain substrates and the ion-dependent activation toward other substrates. It can also account for the somewhat contradictory results reported from different laboratories with regard to the functional role of the sulfhydryl groups in the enzyme. It is suggested that this intrinsic malleability might constitute a molecular basis for modulating the specificity of the enzyme and targeting its activity from one substrate to another in response to intracellular specifier signals.

Chemical modification of the sulfhydryl groups in the catalytic subunit (C)[1] of cAMP-dependent protein kinase was studied in several laboratories (Sugden et al., 1976; Kochetkov, et al., 1976; Bechtel et al., 1977; Peters et al., 1977; Armstrong & Kaiser, 1978; Kupfer et al., 1979). However, the conclusions reached regarding the functional assignment(s) of these groups were significantly different; Sugden et al. (1976) found that the bovine liver enzyme contains one SH group per C subunit and that this sulfhydryl is essential for activity. Kochetkov et al. (1976) showed that the C subunit of the porcine brain enzyme contains three cysteine residues which can be blocked without a substantial effect on the catalytic activity of the enzyme. Bechtel et al. (1977) reported that the binding of 1 mol of *N*-ethylmaleimide per mol of C from rabbit skeletal muscle results in almost complete (>85%) inactivation of the enzyme. Peters et al. (1977) carried out a stoichiometric titration of the sulfhydryl groups in bovine heart C with Nbs_2 and came to the conclusion that inactivation of the enzyme by reaction with this reagent results from the modification of two SH groups. Furthermore, they provided evidence suggesting that none of the SH groups in the enzyme is directly involved in catalysis, since a percyanylated derivative of the enzyme retained 63% of the activity of the enzyme. Working

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[1] Abbreviations: AMP-PNP, adenosine 5′-(β,γ-imidotriphosphate); C, the catalytic subunit of cAMP-dependent protein kinase; cAMP, adenosine cyclic 3′,5′-phosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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with C prepared from the same source, Armstrong & Kaiser (1978) found by kinetic studies that the enzyme possesses two titrable SH groups and that both these groups have to be modified for complete inactivation of the enzyme. We have recently shown (Kupfer et al., 1979) that an affinity labeling reagent originally designed to label the active site of trypsin acts also as an active-site-directed chemical reagent for free C. Using this reagent (N^{α}-tosyl-L-lysyl chloromethyl ketone), we found that in rabbit skeletal muscle C there is one (though not necessarily only one) SH group which is present either at the active site of the enzyme or at another site intimately associated with it (Kupfer et al., 1979) and that modification of this SH group suffices to totally inactivate the enzyme.

Some of the above-mentioned experiments were carried out with C originating from the "peak I" form of the enzyme and some with the "peak II" form. However, this in itself is not likely to account for the discrepancies, since there is considerable evidence in the literature suggesting that the C subunit of the two forms of the enzyme may be very similar, if not identical (Nimmo & Cohen, 1977). On the other hand, the above-mentioned modifications were carried out under different experimental conditions (modifying reagent, pH, ionic strength, ionic composition, etc.), and it was therefore possible that these differences might be responsible, at least in part, for the different results obtained. This paper addresses itself to this question and provides evidence showing that the catalytic subunit of cAMP-dependent protein kinase readily undergoes salt-induced conformational changes, which can be put to use for manipulating at will the relative reactivity and availability of the SH groups of C and for establishing a connection between one (kinetically characterized) SH group and the γ-P subsite in the ATP site of the enzyme. Furthermore, this intrinsic structural malleability of C might reflect a functional (possibly regulatory) assignment of the enzyme.

Materials and Methods

Free Catalytic Subunit of cAMP-Dependent Protein Kinase. The enzyme was obtained from rabbit skeletal muscle in a homogeneous form, following the procedure of Beavo et al. (1974). The peak I form of the undissociated enzyme served as a source for the preparation of C.

Other Materials. Histone H2b was purified by the method of Böhm et al. (1973). The following materials were obtained from commercial sources: [γ-^{32}P]ATP (2.0–2.7 Ci/mmol) was from the Radiochemical Centre, Amersham; ATP, ADP, AMP, AMP-PNP, Hepes, Nbs_2, dithioerythritol, 2,2′-dipyridyl disulfide, bovine serum albumin, and ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid were from Sigma; EDTA was purchased from Fluka; $Mg(CH_3COO)_2$ was from BDH; the buffers 2-(*N*-morpholino)ethanesulfonic acid and 3-(*N*-morpholino)propanesulfonic acid were from Serva. All other chemicals were the best available grade from commercial sources.

Assay of cAMP-Dependent Protein Kinase Activity. The assay was based on the phosphorylation of histone H2b with [γ-^{32}P]ATP as described elsewhere (Kupfer et al., 1979). The enzyme preparations used had a specific activity of 8–13 units/mg (with 1 unit of enzyme activity being defined as the amount of enzyme which catalyzes the transfer of 1 μmol of ^{32}P from [γ-^{32}P]ATP onto histone H2b per min at pH 6.5 and 30 °C). For determination of initial velocities (Figure 6 and Table I), the assay medium used contained 3-(*N*-morpholino)propanesulfonic acid (20 mM), $MgCl_2$ (10 mM), ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*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 s at 30 °C, and in all cases linear incorporation of ^{32}P into histone H2b was ascertained during the 60-s assay. The assay was arrested by applying an aliquot (50 μL) of the reaction mixture on a filter paper that had been soaked in 10% CCl_3COOH and then dried. The ionic strength of the medium was adjusted with NaCl. In the range of NaCl concentrations used, the salt did not affect the precipitation of the histone on the filter papers used in the assay.

Protein Concentration. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference standard. A molecular weight of 40 000 was used for C (Hofmann et al., 1975) in calculating molar concentrations.

Chemical Modification of the Enzyme with Nbs_2. Freshly prepared enzyme solutions were incubated with dithioerythritol (3 mM) for 1 h at 37 °C in order to reduce sulfhydryl groups of the enzyme which might have been oxidized in the course of its isolation. The enzyme solution was then freed of any low molecular weight thiol compounds by gel filtration on a column of Sephadex G-25 (fine) equilibrated and run (at 23 ± 1 °C) with a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0. Only such freshly reduced enzyme solutions were used for chemical modification studies, which were carried out immediately upon collection of the protein fractions emerging from the column. The modification of SH groups was monitored by the formation of the nitrothiobenzoate anion, using a molar absorbancy ϵ_{412nm} of 13 600 M^{-1} cm^{-1} (Ellman, 1959). This molar extinction coefficient was found to be essentially constant in the pH range 7–8. So that the inactivation of the enzyme could be followed, the reaction with Nbs_2 was stopped at the indicated times by removing aliquots of 25 μL from the reaction mixture 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. The presence of diluted Nbs_2, after the reaction was stopped, was found to cause less than 3% inactivation in the course of the manipulations involved. No measurable inactivation could be detected in the controls (without Nbs_2) while the inactivation was monitored.

Analysis of the Kinetic Data. The chemical modification of the enzyme was always carried out under pseudo-first-order conditions (Nbs_2 concentration was over 50-fold higher than the molar concentration of the enzyme). Second-order rate constants were calculated from the linear dependency of the pseudo-first-order rate constants on the concentration of Nbs_2. In the calculation of rate constants for the modification of SH groups in C, corrections were made to take into account the contribution of the slower reacting SH groups according to Ray & Koshland (1961).

Results

Sulfhydryls of the C Subunit as Reporter Groups for the Conformation of the Enzyme. Curve I in Figure 1 represents a typical experiment in which the sulfhydryls of C are modified by Nbs_2 at neutral pH. At about 2.3 mol of SH groups per mol of C, the titration curve levels off. Beyond that point no additional sulfhydryls could be revealed by titration with Nbs_2 even when sodium dodecyl sulfate (2%) was included in the reaction medium (data not shown). Upon plotting the time course of the reaction as pseudo-first-order kinetics (Figure 1B), at least two phases can be distinguished: a slower one with a rate constant $k_1 = (2 \pm 0.3) \times 10^2$ M^{-1} min^{-1}, corresponding to the titration of 0.9 ± 0.1 mol of SH per mol of C, which will be referred to as the class I sulfhydryls (SH_I),

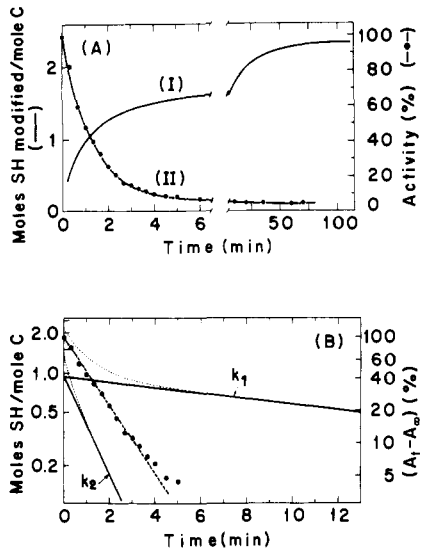

FIGURE 1: Rate of inactivation of C by Nbs_2 compared with the rate of reaction of its sulfhydryl groups with the same reagent. The reaction mixtures (1.05 mL) contained pure C (final concentration 4.8 μM) in a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0. The reaction was initiated by addition 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) (I) 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; (II) time course of the inactivation of the enzyme monitored by assay of aliquots removed at various times as described under Materials and Methods. (Panel B) First-order plots for the inactivation of the enzyme (●) and for the modification of its SH groups by Nbs_2 (···). Activity values were corrected for the residual activity of the fully modified enzyme. The rate constants k_1 and k_2 are calculated from an analysis of curve I (panel A) according to Ray & Koshland (1961).

and a faster phase with a rate constant $k_2 = (3.4 \pm 0.5) \times 10^3$ M^{-1} min^{-1}, corresponding to 1.0 ± 0.1 mol of SH per mol of C, which will be referred to as the class II sulfhydryls (SH_{II}). Extrapolation to time zero of either one of the reaction traces (Figure 1B) suggests the possible existence of an additional, very fast phase, which, unlike those associated with the modification of SH_I and SH_{II}, corresponds to only 0.3 ± 0.2 mol of SH per mol of C. This modification occurs with a rate constant $k_3 > 10^4$ M^{-1} min^{-1}. In view of the fact that after performic acid oxidation and amino acid analysis several investigators have determined three cysteic acid residues per C subunit (Bechtel et al., 1977; Demaille et al., 1977; Armstrong & Kaiser, 1978; Kupfer et al., 1978), the above-mentioned fast phase could be due to the existence of a third class of SH groups (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 titrations with Nbs_2. However, recent sequence studies carried out by Shoji et al. (1981) have clearly shown that at least the C subunit isolated from bovine cardiac muscle contains only two cysteine residues, suggesting that the above-mentioned fast phase might be associated with either an impurity (which could not be revealed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and staining with Coomassie blue) or a unique (unknown yet) structural feature of this enzyme.

When the rate constants k_1 and k_2 were determined in two buffers which differed only in the concentration of added NaCl (changing the ionic strength from 0.03 to 0.22), it was found that both constants were considerably altered. However, they were unexpectedly altered in opposing directions: while k_2 decreased from 3400 (at ionic strength 0.03) to 900 M^{-1} min^{-1} (at ionic strength 0.22), i.e., by ~3.8-fold (Figure 2B), k_1 increased from 200 to 900 M^{-1} min^{-1}, i.e., by ~4.5-fold (Figure 2A). The overall effect is clearly illustrated in Figure 2C which shows how the ratio k_2/k_1 dramatically decreases (over 16-fold) within a rather narrow range of ionic strengths around physiological values.

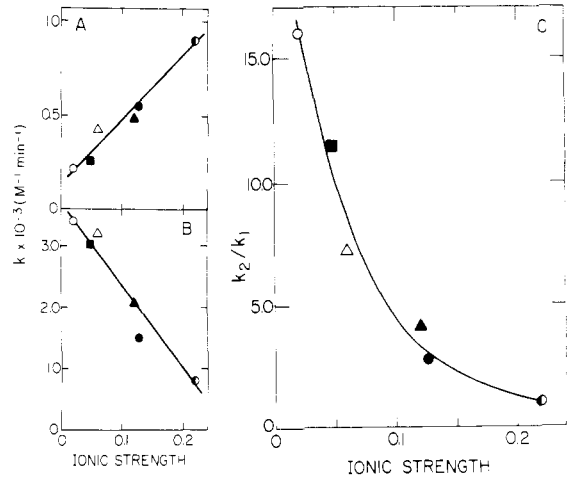

FIGURE 2: Effect of ionic strength on the rate constants k_1 and k_2 and on the ratio k_2/k_1. The reaction mixtures (pH 7.0; 23 ± 1 °C) included the following: (a) C subunit (4 μM), Hepes (0.1 M), and EDTA (1 mM) (○); (b) same as (a) plus NaCl (0.1 M) (●); (c) same as (a) plus NaCl (0.2 M) (◐); (d) C subunit (2.2 μM), Hepes (50 mM), EDTA (1 mM), and NaCl (30 mM) (■); (e) C subunit (3.5 μM), Hepes (0.1 M), EDTA (1 mM), and $Mg(CH_3COO)_2$ (12 mM) (△); (f) C subunit (5.7 μM), potassium phosphate (50 mM), and EDTA (1 mM) (▲). Panel A depicts the effect of ionic strength on k_1, panel B depicts the effect of ionic strength on k_2, and panel C illustrates the effect of ionic strength on the ratio k_2/k_1. 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 Materials and Methods.

Since the rate of chemical modification of low molecular weight sulfhydryl compounds (such as glutathione) is not affected to that extent within the narrow range of ionic strengths used here (Warren & Seatum, 1966) and especially since the reactivity toward Nbs_2 of the two sulfhydryl classes (SH_I and SH_{II}) is affected by the change in ionic strength in opposite directions (Figure 2A,B), 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, for example, in their steric availability, surrounding dielectric constant, vicinal interacting groups, etc. In other words, the enzyme seems to undergo a pronounced conformational change upon increasing the ionic strength from 0.03 to 0.22, which is clearly reflected in the relative reactivity of SH_I and SH_{II}.

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 used 2,2'-dipyridyl disulfide, an analogue of Nbs_2 which has no net charge under the conditions of these experiments (the pK_a of the pyridinium nitrogen of this analogue was reported to be 2.45; Brocklehurst & Little, 1973). This comparison was carried out by monitoring the inactivation of the enzyme by either one of the two reagents at different salt concentrations. It was found that the inactivation of the enzyme by both the negatively charged Nbs_2 and its neutral analogue was similarly affected upon changing the ionic strength (not illustrated), in

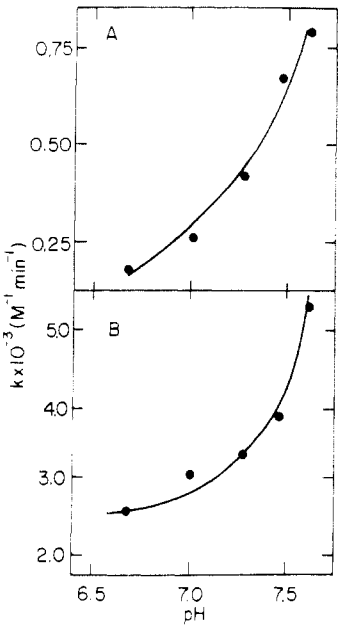

FIGURE 3: Effect of pH on the rate constants k_1 and k_2. The reaction mixtures (1.06 mL) contained C (2.2 μM), Hepes (50 mM), EDTA (1 mM), NaCl (keeping the ionic strength constant at 0.045), and Nbs_2 (120 μM). The rate constants k_1 (panel A) and k_2 (panel B) were determined as described under Materials and Methods (pH 7.0; 23 ± 1 °C).

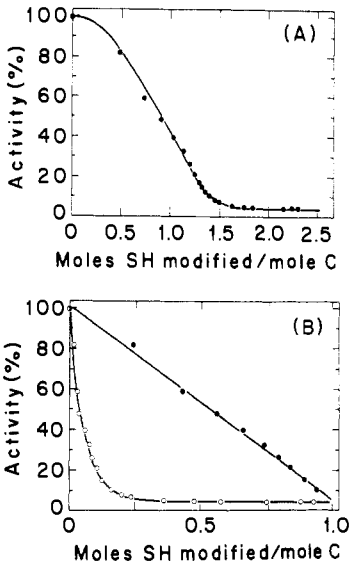

FIGURE 4: Stoichiometry of the inactivation of C by Nbs_2. (Panel A) Residual activity of the enzyme as a function of the overall number of SH groups modified per mole of C subunits. (Panel B) Residual activity of the enzyme as a function of the extent of modification of sulfhydryls reacting with a rate constant k_1 (○) and of sulfhydryls reacting with a rate constant k_2 (●). The reaction mixtures and experimental procedures were identical with those described in the legend to Figure 1.

agreement with our suggestion that the effect of ionic strength 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 Figure 2 were carried out in different ionic compositions of the medium, yet both k_1 and k_2 (and thus their ratio k_2/k_1) were not affected by the specific ionic composition. Therefore, it seems likely that this conformational change (or "deformation"; cf. Shaltiel et al., 1966; Hedrick et al., 1969) is ionic strength dependent rather than ion specific.

As expected, the rate constants k_1 and k_2 are also affected by the pH of the medium in which the reaction with Nbs_2 is carried out. However, in contrast to the effect of ionic strength, changing the pH affected both k_1 and k_2 in the same direction, with both constants increasing with increasing pH (Figure 3). This pH-dependent increase in both constants probably arises from the ionization of the SH groups, which are known to react with Nbs_2 around neutrality via a nucleophilic attack of the thiolate ion (Brocklehurst & Little, 1973).

While both k_1 and k_2 increase upon increasing the pH, it should be emphasized that the ratio k_2/k_1, i.e., the relative reactivities of SH_{II} and SH_I, changes from 13.9 to 6.8 within less than 1 pH unit around neutrality. Therefore, the differences reported from different laboratories regarding the rates of modification of the various sulfhydryls in the enzyme (and the stoichiometry of such inactivations; see below) may well be due, at least in part, to differences in reaction conditions (such as pH and ionic strength) under which these experiments were carried out since, as shown here, the ratio k_2/k_1 can vary from 1 (where the two classes of sulfhydryls are modified at the same rate) to ~16 (where one sulfhydryl class is practically unaffected while the other one is being modified). On the other hand, the fact that the relative reactivity of SH_{II} and SH_I is so sensitive to structural changes in the enzyme makes these functional groups very useful as built-in reporter groups for the conformation of the enzyme.

The Inactivation of C by Nbs_2 Occurs Concomitantly and Stoichiometrically with the Modification of SH_{II}. When the time course of the inactivation of C by Nbs_2 was plotted in a first-order kinetics plot (Figure 1B), a rate constant $k_{inact} = (3 \pm 0.5) \times 10^3$ M^{-1} min^{-1} was determined, which is very close to the rate constant (k_2) determined for the modification of the class II sulfhydryls [$k_2 = (3.4 \pm 0.5) \times 10^3$ M^{-1} min^{-1}; Figure 1B]. It should be noted that toward the completion of the inactivation process depicted in Figure 1B, there is some deviation from linearity. This deviation could arise from the possible occurrence of an additional inactivation process which could involve, for example, the class I sulfhydryls. However, under the conditions of our experiment, this process could account for less than 6% of the inactivation of the enzyme.

Figure 4A depicts the residual activity of the enzyme as a function of the number of moles of SH groups modified by Nbs_2 per mole of C. This titration curve clearly shows that, after an initial lag (up to ~0.3 mol of SH groups/mol of C, which are very reactive yet unessential for activity), the modification of ~1 additional mol of SH groups brings about ~90% of inactivation. The next mole of reagent reacting with the enzyme causes an additional inactivation amounting to ~6% only (Figure 4A).

Since the reactivities of the various classes of sulfhydryls are so easily resolved at low ionic strength, it is possible to quantitatively determine under these conditions the extent of modification of each one of these groups during the course of enzyme inactivation. As seen in Figure 4B, SH_{II} can certainly be regarded as essential for activity. This is clearly evident from the occurrence of a linear stoichiometric relationship between the inactivation of the enzyme and the modification of this class of sulfhydryls. In other words, there is a quantitative relationship between the fraction of SH_{II} modified at each stage and the percentage of enzyme inactivation. This is in good agreement with the finding of Bechtel et al. (1977) that the binding of 1 mol of *N*-ethylmaleimide per mol of C (from rabbit skeletal muscle) at neutral pH and low ionic strength (10 mM phosphate) inhibits more than 85% of the activity of the enzyme.

In spite of the above, it should be emphasized that although (under the conditions of the experiment depicted in Figure 4) the inactivation of the enzyme is shown to occur concomitantly

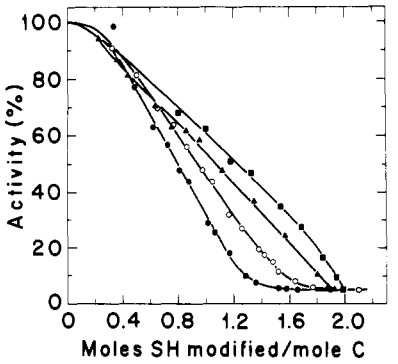

FIGURE 5: Effect of ionic stength on the stoichiometry of the inactivation of C by Nbs_2. The reaction mixtures included the following: (a) C subunit (4 μM), Hepes (0.1 M), and EDTA (1 mM) (●); (b) same as (a) plus $Mg(CH_3COO)_2$ (12 mM) (○); (c) same as (a) plus NaCl (0.1 mM) (▲); (d) same as (a) plus NaCl (0.2 M) (■). The reaction was initiated in each case by addition of Nbs_2 (to a final concentration of 215 μM). It was allowed to proceed at pH 7.0 and 23 ± 1 °C. The set of points in each curve was obtained by following simultaneously the inactivation of the enzyme and the modification of SH groups as described under Materials and Methods.

and stoichiometrically with the modification of the class II sulfhydryls, this, in itself, does not rule out the possibility that the class I sulfhydryls may also be essential for activity, since under these conditions the inactivation of the enzyme is practically completed before the class I sulfhydryls start to be modified.

In order to estimate the maximal extent of inactivation that could have been achieved by the modification of SH_I alone, we took advantage of our ability to vary the ratio k_2/k_1 by merely changing the concentration of added NaCl. As seen in Figure 5, upon increasing the ionic strength, the stoichiometry of the inactivation is significantly affected. This is presumably due to the fact that under these conditions the class I and class II sulfhydryls are modified at comparable rates and the modification of each one of these sulfhydryl classes might then be responsible for part of the inactivation. For example, in the presence of 0.19 M NaCl the modification of the SH groups of C becomes monophasic with a common rate constant (for both k_1 and k_2) of 900 M^{-1} min^{-1}. We have shown above that the modification of SH_{II} alone can bring about an almost quantitative (~90%) inactivation. If the modification of SH_I alone would also result in a practically inactive enzyme, then the rate constant of inactivation should have been the sum of k_1 and k_2, namely, k_{inact} = ~1800 M^{-1} min^{-1}. However, the rate constant of inactivation was found to be only 800 M^{-1} min^{-1}, indicating that the modification of SH_I could not fully inactivate the enzyme. In fact, theoretical analysis of the inactivation curve according to Ray & Koshland (1961) shows that if complete modification of the class I sulfhydryls could in itself bring about a partial inactivation of the enzyme, this inactivation would certainly not exceed 40%.

Kinetic Analysis of the Salt-Induced Inactivation of C When Histone H2b Is Used as a Substrate. The fact that the phosphorylation of histones (or casein) by cAMP-dependent protein kinase is inhibited upon increasing the ionic strength of the assay medium was observed in several laboratories (Jergil & Dixon, 1970; Reimann et al., 1971; Walsh & Krebs, 1973; Moll & Kaiser, 1977; Zick et al., 1979; Palmer et al., 1980). In principle, this could be due to an effect of ionic strength either on these particular protein substrates or on the enzyme itself, or on both. In fact, since some authors have found that increasing the ionic strength can sometimes have an inhibitory effect and sometimes a stimulatory effect, dependent on the nature of the protein substrate used, they suggested that the ionic strength effect is primarily on the substrate rather than on the enzyme itself (Walsh & Krebs, 1973; Singh & Wang, 1977). 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, strongly indicates that the inactivation might well 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 (Cleland, 1970)

$$v = \frac{VAB}{K_{AB} + AK_B + BK_A + AB} \quad (1)$$

where A and B stand for the concentrations of the substrates (in this case MgATP and histone H2b, respectively), V is the maximal velocity, K_A and K_B are the Michaelis constants with respect to A and B, and K_{AB} is a constant defined by Alberty (1953).

The kinetic constants can be determined from secondary plots of the double-reciprocal presentations of the initial rate as a function of the concentration of one substrate (A or B) at varying concentrations of the other substrate (B or A, respectively) by using eq 2 (where B is constant) and eq 3 (where A is constant) derived from eq 1.

$$\frac{1}{v_b} = \frac{1}{A}\left(\frac{K_{AB}}{VB} + \frac{K_A}{V}\right) + \frac{1}{B}\left(\frac{K_B}{V}\right) + \frac{1}{V} \quad (2)$$

$$\frac{1}{v_a} = \frac{1}{B}\left(\frac{K_{AB}}{VA} + \frac{K_B}{V}\right) + \frac{1}{A}\left(\frac{K_A}{V}\right) + \frac{1}{V} \quad (3)$$

The intercepts of these plots derived from eq 2 and 3 are given in eq 4 and 5, respectively.

$$\text{intercept}_b = \frac{1}{B}\left(\frac{K_B}{V}\right) + \frac{1}{V} \quad (4)$$

$$\text{intercept}_a = \frac{1}{A}\left(\frac{K_A}{V}\right) + \frac{1}{V} \quad (5)$$

From the measured intercepts in the above-mentioned plots (Figure 6) and eq 4 and 5, one can readily calculate the maximal velocity (V) as well as the Michaelis constants for MgATP (K_A) and histone H2b (K_B). 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 I, 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 Michealis 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 the affinity of the enzyme toward its substrates.

Interestingly, both Michaelis constants (K_A and K_B) increase similarly (by 3.4–4-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 ag-

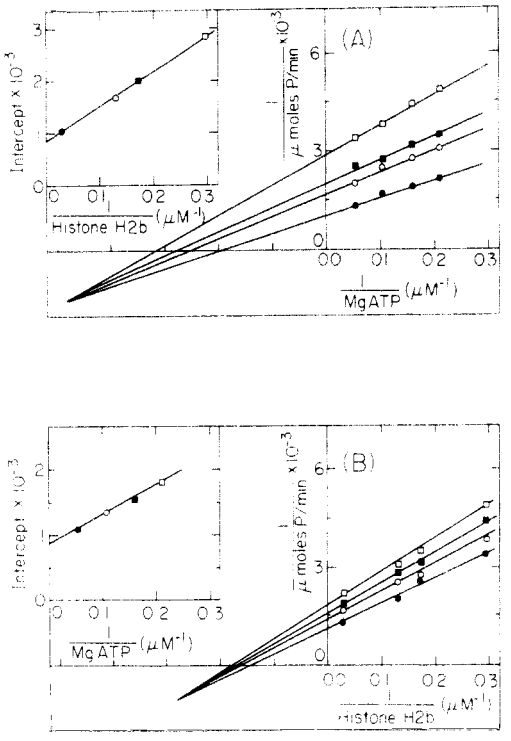

FIGURE 6: Plots of reciprocals of steady-state initial velocities vs. reciprocals of substrate concentrations for the reaction catalyzed by cAMP-dependent protein kinase. In addition to the enzyme (60 ± 10 ng) and the buffer described under Materials and Methods, the assay mixtures contained, in panel A, the indicated concentration of MgATP and the following concentrations in histone H2b (μM); 34.8 (●), 7.8 (○), 5.8 (■), and 3.4 (□). Similarly, in panel B the assay mixtures contained the indicated concentration of histone H2b and the following concentrations of MgATP (μM): 17.5 (●), 9.4 (○), 6.3 (■), and 4.7 (□). Insets represent secondary plots, used for the calculation of the maximal velocity V and Michaelis constants for ATP (K_A) and for histone H2b (K_B).

Table I: Effect of NaCl Concentration on the Maximal Velocity and the Michaelis Constants of the Enzyme[a]

[NaCl] (M)	maximal velocity (V) (nmol of γ-^{32}P incorporated/min)	Michaelis constants for ATP (K_A) (μM)	Michaelis constants for histone H2b (K_B) (μM)
	1.2 ± 0.2	5 ± 1	8 ± 1
0.03	1.6 ± 0.3	15 ± 4	17 ± 5
0.11	1.6 ± 0.4	17 ± 3	32 ± 9

[a] The kinetic parameters were obtained from experiments similar to those described in Figure 6 and calculated from the equations given in the text.

gregation), 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.

The fact that the lines in the double-reciprocal plots depicted in Figure 6 converge is consistent with a sequential mechanism whereby both substrates have to be bound to the enzyme prior to the release of products. Although it is not possible to deduce from the above whether the order of binding of the two substrates is random or predetermined, the kinetic experiments described here imply the existence of two independent sites, one for accommodating MgATP and another for histone H2b. Since the inactivation of the enzyme occurs concomitantly and stoichiometrically with the modification of its class II sulfhydryls, it seems reasonable to assume that SH_{II} is likely to be structurally associated with the active site if it does not

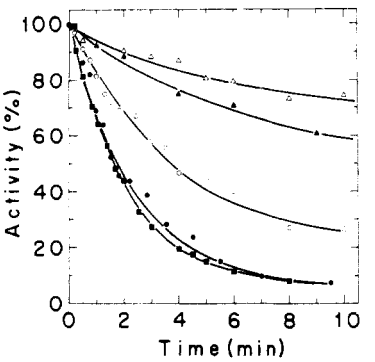

FIGURE 7: Effect of nucleotides and Mg^{2+} on the kinetics of inactivation of C by Nbs_2. The reaction mixtures included the following: (a) C subunit (3.5 μM), Hepes (0.1 M), EDTA (1 mM), $Mg(CH_3COO)_2$ (12 mM), and Nbs_2 (190 μM) (■); (b) C subunit (2.9 μM), Hepes (0.1 M), EDTA (1 mM), $Mg(CH_3COO)_2$ (50 mM), AMP (31 mM), and Nbs_2 (150 μM) (●); (c) C subunit (3 μM), Hepes (0.1 M), EDTA (1 mM), $Mg(CH_3COO)_2$ (21 mM), ADP (4 mM), and Nbs_2 (160 μM) (○); (d) C subunit (3.4 μM), Hepes (0.1 M), EDTA (1 mM), $Mg(CH_3COO)_2$ (11 mM), AMP-PNP (5.5 mM), and Nbs_2 (190 μM) (▲); (e) C subunit (6 μM), Hepes (0.1 M), EDTA (1 mM), $Mg(CH_3COO)_2$ (4.5 mM), ATP (1.1 mM), and Nbs_2 (340 μM) (△). All the reactions were initiated by addition of Nbs_2 (which was in all cases in ~50-fold molar excess over the enzyme) and allowed to proceed at pH 7.0 (23 ± 1 °C). The residual activity was monitored as described under Materials and Methods with appropriate controls in which buffer alone was added instead of the Nbs_2 solution.

reside at the active site itself. It is interesting, therefore, to establish with which one of the two sites this sulfhydryl is connected and try to pinpoint the location of this group in relation to this site.

Establishing a Connection between SH_{II} and the γ-P Subsite in the ATP Site of the Enzyme. In an attempt to find out if the class II sulfhydryls are associated with the nucleotide site of the enzyme, we investigated the effect of MgATP and some of its analogues, which are known to bind to this site (Hoppe et al., 1978), on the kinetics of modification of the enzyme by Nbs_2. It was found that the presence of MgATP strongly interferes with the modification of the class II sulfhydryls. The time course of the reaction fits a monophasic process with a rate constant $k = 1.7 \times 10^2$ M^{-1} min^{-1} (not illustrated), indicating that the class II sulfhydryls become much less reactive, while class I sulfhydryls are essentially unaffected.

Since the modification of the class II sulfhydryls brings about inactivation of the enzyme, one could expect MgATP to afford also a protection from inactivation by Nbs_2. This is indeed the case; not only does MgATP afford protection but it does so at concentrations (≥10 μM) which correspond to the K_m value of the enzyme for this nucleotide substrate. The inactivation of the enzyme in the presence of an excess of MgATP (100 times its K_m) is about 40 times slower than in the absence of the substrate ($k^{ATP}_{inact} = 80$ M^{-1} min^{-1}). The presence of $Mg(CH_3COO)_2$ alone does not protect the enzyme from inactivation at all (Figure 7).

It is interesting to note which ones among a number of ATP analogues are capable of affording protection from inactivation. In the series ATP, AMP-PNP, ADP, and AMP, a significant protection was observed only with the two nucleotides containing the γ-P group (ATP and AMP-PNP). In the absence of the γ-P (ADP), the protection was considerably lower, and absence of the β-P as well (AMP) resulted in an analogue that afforded no protection from inactivation whatsoever (Figure 7).

The possibility that the extent of protection mentioned above merely reflects the different affinities of the enzyme for the

various ATP analogues (Hoppe et al., 1978) could be ruled out, since in all experiments an appropriate excess of the ATP analogue was used (100 times its K_i value, which for such analogues is apparently close to the analogous K_d values; Feramisco, 1978; Armstrong et al., 1979). Additionally, the concentration of $Mg(CH_3COO)_2$ was always such as to keep more than 50% of the ATP analogue in the form of its magnesium complex (in the bulk solution), according to the association constant of these complexes (Tu & Heller, 1974) and of $Mg(CH_3COO)_2$ (Nancollas, 1956). Moreover, in spite of having a higher K_i than ADP (cf. Hoppe et al., 1978), AMP-PNP was found to afford a much more pronounced protection from inactivation by Nbs_2 than ADP does.

It is noticeable that the increasing ability of the various ATP analogues to afford protection against inactivation is concomitant with a gradual decrease of the reactivity of class II sulfhydryls toward Nbs_2 in the presence of those nucleotides ($k = 3.2 \times 10^3$, 1.7×10^3, 4.5×10^2, and 1.7×10^2 M^{-1} min^{-1} for AMP, ADP, AMP-PNP, and ATP, respectively) whereas the reactivity of class I sulfhydryls was not significantly altered.

It seems therefore that the γ-P group of ATP is essential for affording protection to the enzyme from its inactivation by Nbs_2 and that there is a connection between the class II sulfhydryl and the γ-P subsite of the ATP binding site in the enzyme. The sulfhydryl could therefore be either at this subsite or at another site which is structurally associated with it.

Discussion

The results presented in this paper suggest that the free catalytic subunit of cAMP-dependent protein kinase undergoes pronounced, salt-induced conformational changes which can be monitored directly by the relative chemical reactivity of the SH groups of the enzyme, which act here as built-in sensitive reporter groups. It is shown that by manipulating the ionic strength of the medium it is possible to change at will the relative reactivity of the different sulfhydryls in the enzyme. This enables us to distinguish between "essential" and "nonessential" sulfhydryl groups in the enzyme (an essential sulfhydryl group being defined as an SH whose chemical modification brings about an inactivation of the enzyme). These results also set the stage for the preparation of homogeneously labeled enzyme derivatives, which are potentially useful for X-ray studies and for establishing structure–function relationships. By use of such manipulations on the one hand and ATP analogues on the other, evidence was obtained suggesting that one (kinetically characterized) sulfhydryl group is essential for the activity of the enzyme and that it is associated with the γ-P subsite in the ATP binding site of the catalytic subunit of the kinase. At first glance, it seems that this conclusion cannot be reconciled with the results of Peters et al. (1977), who found that percyanylation of C (incorporation of 3.0 mol of CN groups per mol of C) yields an enzyme which exhibits 63% of the activity of the native enzyme, suggesting that neither of the sulfhydryls is really indispensible for activity. However, even if we assume that in this experiment SH_{II} is among the groups that are modified and that this modification is retained during the assay of the enzyme, it should be realized that these findings of Peters et al. (1977) would exclude the possibility that SH_{II} is involved directly in the catalytic mechanism, say as an $-S^-$ or as a proton donor, but it would not exclude the possibility that SH_{II} is located at the ATP binding site playing another (catalytic or recognition) role, which the –SCN derivative is able to fulfill with 63% efficiency.

It should be emphasized, however, that each of the above observations by itself might also be accounted for if SH_{II} were to be placed at another location in the enzyme molecule, a location which would be distal to the active site but structurally linked with it. For this to be the case, we would have to assume that the γ-P of ATP upon binding to the active site triggers a conformational change in C that makes SH_{II} much less available to chemical reaction. In addition, we would have to assume that a covalent modification by Nbs_2 of SH_{II} alone suffices to trigger a conformational change which is reflected at the active site, bringing about inactivation of the enyzme.

While we cannot, at this stage, completely dismiss the occurrence of such conformational changes, it seems that placing SH_{II} at the active site itself accounts for the above observations and also for those described in other publications from our laboratory (Kupfer et al., 1979, 1980, Jiménez et al., 1981; cf. Kupfer et al., 1982).

The salt-induced conformational changes of C, 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 (K_m) for this substrate without impairing the potential catalytic activity of the enzyme, measured by its maximal velocity (V_{max}).

Being an intrinsic property of the enzyme, the structural malleability of the C subunit might account (at least in part) for the well-known in vitro salt inhibition of the catalytic activty of this enzyme toward certain substrates (Jergil & Dixon, 1970; Walsh & Krebs, 1973; Moll & Kaiser, 1977; Zick et al., 1979; Palmer et al., 1980), as well as for the fact that upon binding certain ions (e.g., Mg^{2+}) the enzyme may become activated toward other substrates (Singh & Wang, 1977). 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 (Kochetkov et al., 1976; Sugden et al., 1976; Bechtel et al., 1977; Peters et al., 1977; Armstrong & Kaiser, 1978; Kupfer et al., 1979).

Generally speaking, changes in ionic strength from 0.03 to 0.22 are not known to occur under in vivo conditions, and therefore it is not suggested here that the ionic strength sensitivity of C constitutes a physiological mechanism for regulation. However, the intrinsic structural malleability of this enzyme (which is illustrated here by its remarkable sensitivity to slight changes in ionic strength) can very well lend itself to the action of physiological inhibitors (Walsh et al., 1971; Demaille et al., 1977; Hashimoto et al., 1979) and modulators (Donnelly et al., 1973a,b; Gagelmann et al., 1980) of this enzyme, which may regulate the action of C even after it has been released from its "stored" form (undissociated cAMP-dependent protein kinase). It might be inferred from the foregoing that C is capable of assuming different conformations, with probably different specificities, and thus has its activity diverted from one substrate to another, where and when the need arises. Recent reports from several laboratories (cf., of example, Hayes et al., 1980) indicate that the specific response of cells to hormones functioning via cAMP probably does not depend only on the hormone receptors on the cell surface and the substrates available for protein kinase in a given cell. The malleability of the C subunit, if designed to detect additional specifier signals in vivo, might thus offer an additional device for regulation.

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References

Alberty, R. A. (1953) *J. Am. Chem. Soc. 75*, 1928.
Armstrong, R. N., & Kaiser, E. T. (1978) *Biochemistry 17*, 2840.
Armstrong, R. N., Kondo, H., & Kaiser, E. T. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 722.
Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1974) *Methods Enzymol. 38*, 299.
Bechtel, P. J., Beavo, J. A., & Krebs, E. G. (1977) *J. Biol. Chem. 252*, 2691.
Böhm, E. L., Strickland, M., Thwaits, B. H., van der Westhuzen, D. R., & Von Holt, G. (1973) *FEBS Lett. 34*, 217.
Brocklehurst, K., & Little, G. (1973) *Biochem. J. 133*, 67.
Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2*, 1.
Demaille, J. G., Peters, K. A., & Fischer, E. H. (1977) *Biochemistry 16*, 3080.
Donnelly, T. E., Jr., Kuo, J. F., Reyes, P. L., Liu, Y.-P., & Greengard, P. (1973a) *J. Biol. Chem. 248*, 190.
Donnelly, T. E., Jr., Kuo, J. F., Miyamoto, E., & Greengard, P. (1973b) *J. Biol. Chem. 248*, 199.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.
Feramisco, J. R. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1328.
Gagelmann, M., Reed, J., Kübler, D., Pyerin, W., & Kinzel, V. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2492.
Hashimoto, E., Kuroda, Y., Ku, Y., & Nishizuka, Y. (1979) *Biochem. Biophys. Res. Commun. 87*, 200.
Hayes, J. S., Brunton, L. L., & Meyer, S. E. (1980) *J. Biol. Chem. 255*, 5113.
Hedrick, J. L., Shaltiel, S., & Fischer, E. H. (1969) *Biochemistry 8*, 2422.
Hofmann, F., Beavo, J. A., Bechtel, P. J., & Krebs, E. G. (1975) *J. Biol. Chem. 250*, 7795.
Hoppe, J., Freist, W., Marutzky, R., & Shaltiel, S. (1978) *Eur. J. Biochem. 90*, 427.
Jergil, B., & Dixon, G. H. (1970) *J. Biol. Chem. 245*, 425.
Jiménez, J. S., Kupfer, A., Gottlieb, P., & Shaltiel, S. (1981) *FEBS Lett. 130*, 127.
Kochetkov, S. N., Nesterova, M. V., Sashchenko, L. P., & Severin, E. S. (1976) *Biokhimiya (Moscow) 41*, 1127.
Kupfer, A., Hoppe, J., Gani, V., & Shaltiel, S. (1978) *Proc. Annu. Meet. Israeli Biochem. Soc.*, 8.
Kupfer, A., Gani, V., Jiménez, J. S., & Shaltiel, S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3073.
Kupfer, A., Jiménez, J. S., & Shaltiel, S. (1980) *Biochem. Biophys. Res. Commun. 96*, 77.
Kupfer, A., Jiménez, J. S., Gottlieb, P., & Shaltiel, S. (1982) *Biochemistry* (following paper in this issue).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Moll, G. W., Jr., & Kaiser, E. T. (1977) *J. Biol. Chem. 252*, 3007.
Nancollas, G. N. (1956) *J. Chem. Soc.*, 744.
Nimmo, H. G., & Cohen, P. (1977) *Adv. Cyclic Nucleotide Res. 8*, 145.
Palmer, W. K., McPherson, J. M., & Walsh, D. A. (1980) *J. Biol. Chem. 255*, 2663.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691.
Ray, W. J., Jr., & Koshland, D. E., Jr. (1961) *J. Biol. Chem. 236*, 1973.
Reimann, E. M., Walsh, D. A., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1986.
Shaltiel, S., Hedrick, J. L., & Fischer, E. H. (1966) *Biochemistry 5*, 2108.
Shoji, S., Parmelee, D. C., Wade, R. D., Kumar, S., Ericsson, L. H., Walsh, K. A., Neurath, H., Long, G. O., Demaille, J. G., Fischer, E. H., & Titani, K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 848.
Singh, T. J., & Wang, J. H. (1977) *J. Biol. Chem. 252*, 625.
Sugden, P. H., Holladay, L. A., Reimann, E. M., & Corbin, J. D. (1976) *Biochem. J. 159*, 409.
Tu, A. T., & Heller, M. J. (1974) *Met. Ions Biol. Syst. 1*, 2.
Walsh, D. A., & Krebs, E. G. (1973) *Enzymes, 3rd Ed. 8*, 555.
Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1977.
Warren, J. C., & Seatum, S. G. (1966) *Biochemistry 5*, 1702.
Zick, Y., Cesla, R., & Shaltiel, S. (1979) *J. Biol. Chem. 254*, 879.