On the Protein Accommodating Site of the Catalytic Subunit of Adenosine Cyclic 3′,5′-Phosphate Dependent Protein Kinase†

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ABSTRACT: The catalytic subunit (C) of cAMP-dependent protein kinase (EC 2.7.1.37) contains two kinetically characterized sulfhydryl groups SH_I and SH_{II}, one of which (SH_{II}) was previously shown to be intimately associated with the γ-P subsite of the ATP binding site in C [Jiménez, J. S., Kupfer, A., Gani, V., & Shaltiel, S. (1982) *Biochemistry* (preceding paper in this issue)]. In the presence of either histone H2b or protamine (two protein substrates of the enzyme) both SH_I and SH_{II} display a considerably enhanced reactivity toward the negatively charged thiol reagent 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), with a parallel acceleration in the rate of inactivation of the kinase. By use of a series of neutral or negatively charged analogues of Nbs_2, it is shown that the enhanced chemical reactivity of the sulfhydryls occurs only with the negatively charged analogues and therefore it presumably originates from an increase in the local concentration of the reagent around the sulfhydryls, i.e., from a "channeling effect" caused by the positively charged protein substrate. In contrast, the heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly enhances the chemical reactivity of SH_{II} only, without affecting SH_I, but still accelerates the rate of inactivation of the enzyme. Being a good substrate of small size, the channeling effect of this peptide is limited to the active site and its vicinity, suggesting that SH_{II} is adjacent to the protein binding moiety of the active site. This suggestion is further supported by the finding that the regulatory subunit (R) which is known to block access to the active site in the holoenzyme affords a considerable protection from modification to the SH_{II} group of C and preserves the potential catalytic activity of R_2C_2. Furthermore, full shielding of SH_{II} and complete prevention of the loss of potential catalytic activity are achieved by addition of MgATP which (upon binding to its high-affinity site) locks the enzyme in its undissociated form. The implications of these results in terms of the position of SH_{II} within the active site of the enzyme and relative to the position of SH_I are discussed.

The manner by which cAMP[1]-dependent protein kinase interacts in vivo with its various substrates is an intriguing property of this enzyme. Originally discovered by Walsh et al. (1968), this enzyme is known to be composed of two types of subunits, one being catalytically active (C) and the other having a regulatory function (R) (Brostrom et al., 1970; Gill & Garren, 1970; Kumon et al., 1970; Tao et al., 1970; Reimann et al., 1971; Erlichman et al., 1971). These two subunits are assembled together to yield the inactive form of the enzyme (R_2C_2) which is now believed to be activated by cAMP according to

$$R_2C_2 + 4cAMP \rightleftarrows R_2(cAMP)_4 + 2C$$

(Corbin et al., 1978; Weber et al., 1979; Weber & Hilz, 1979; Builder et al., 1980).

In a recent study from this laboratory (Jiménez et al., 1982), it was shown that modification of one (kinetically characterized) sulfhydryl group (SH_{II}) in the free catalytic subunit (C) brings about a concomitant and stoichiometric inactivation of the enzyme and that the rates of these two processes are dramatically attenuated by the presence of either ATP or adenosine 5′-(β,γ-imidotriphosphate). In the absence of the γ-P (as in ADP) the protection afforded is much lower, and in the absence of both γ-P and β-P (as in AMP) there is no protection from inactivation whatsoever (Jiménez et al., 1982). On the basis of these results, it could be concluded that SH_{II} is at least structurally associated with the γ-P subsite of the ATP binding site in C, if not an integral part of it.

Since the catalytic function of this enzyme is to transfer the γ-P from ATP onto a target serine residue in the protein substrate, it would be reasonable to asssume that the subsite accommodating these two substrates is vicinal in the three-dimensional structure of the enzyme and therefore that the presence of protein substrates would affect the reactivity of SH_{II} and thus also the inactivation of the enzyme by Nbs_2. By use of protein and peptide substrates, as well as a series of Nbs_2 analogues, this paper attempts to elucidate the spatial relationship between SH_{II} and the subsites which accommodate ATP and the sequence of amino acids around the target serine residue in the protein substrate.

Materials and Methods

Enzymes. The undissociated cAMP-dependent protein kinase (type I) and its free C subunit were obtained from rabbit skeletal muscle in a homogeneous form, following the procedure of Beavo et al. (1974).

Other Materials. Histone H2b was purified to homogeneity by the method of Böhm et al. (1973). The peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly was synthesized as described by Kemp et al. (1977). Its purity was established by high-voltage paper electrophoresis at pH 1.9, 3.5, and 6.5 by using in each case ninhydrin as well as Sakaguchi sprays to identify the

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[1] Abbreviations: cAMP, adenosine cyclic 3′,5′-phosphate; C, catalytic subunit of cAMP-dependent protein kinase; R, regulatory subunit of cAMP-dependent protein kinase; R_2C_2, cAMP-dependent protein kinase in its undissociated (inactive) form; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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peptide. The amino acid composition of the peptide was determined after hydrolysis (5.7 N HCl, 110 °C, 24 h) by using a Beckman 121 automatic amino acid analyzer. Its composition was found to be the following: Arg, 2.1; Ala, 1.0; Gly, 1.0; Leu, 2.02; Ser, 0.98 (value obtained by hydrolysis for different time intervals and extrapolation). Pure histone H2b, protamine, and the synthetic peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly were shown to be free of any impurity containing titrable sulfhydryls.

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, cAMP, Nbs_2, Hepes, dithioerythritol, 2,2′-dithiodipyridine, 2,2′-dithiobis(5-nitropyridine), 6,6′-dithiodinicotinic acid, 4,4′-dithiodipyridine, and bovine serum albumin were from Sigma; EDTA was purchased from Fluka; Mes was obtained from Serva; $Mg(CH_3COO)_2$ and protamine (Clupeine sulfate) were from BDH; 4,4′-dithiobis(3-nitrobenzoic acid) was a gift of Professor M. Fridkin.

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). R_2C_2 was assayed in the presence of cAMP in a final concentration of 5 μM. 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.

Protein Concentrations. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a reference standard. Molecular weights of 40 000 and 48 000 were used for C and R subunits, respectively (Hofmann et al., 1975), 13 800 was used for histone H2b (DeLange & Smith, 1971), and 4250 was used for protamine (Ando et al., 1973).

Chemical Modifications. The chemical modifications of the thiol residues of the enzyme and the kinetic analysis of the data were carried out as described in the preceding paper (Jiménez et al., 1982). In the case of the R_2C_2 form of the enzyme (which contains a large number of titrable SH groups), the kinetic data were analyzed by the graphical method of Ray & Koshland (1961) and also by fitting to a sum of exponents using a computer program (IBM 370/165) with a modified Marguardt Subroutine of the Harwell Library (Fletcher, 1971) (the latter was kindly carried out by Dr. Y. Blatt). The number of exponential terms was chosen so as to give the smallest standard deviation and a stoichiometrically significant number of SH groups corresponding to each exponential term. The results obtained by the two methods were in good agreement (within 10%). It should be noted that in this case the thiol classes display considerably different, and thus easily resolvable, reactivities.

Results and Discussion

Histone H2b Accelerates the Inactivation of C by Nbs_2 and the Concomitant Chemical Modification of both SH_I and SH_{II}. In contrast to ATP (the nucleotide substrate of C) which dramatically attenuates the rate of inactivation of the enzyme by Nbs_2, histone H2b (a protein substrate of C) brings about an opposite effect, considerably accelerating (~5-fold) this inactivation (Figure 1, panels A and C). As seen in Table I, histone H2b (18–72 μM) also accelerates the rate of chemical modification of the enzyme by Nbs_2. However, unlike ATP (which exerts its protective effect on SH_{II} only), histone H2b accelerates the rate of modification of both SH_I and SH_{II} (cf. panels B and D in Figure 1), making the kinetics of the reaction monophasic. This acceleration occurs also with protamine (Table I), another well-known protein substrate of the kinase.

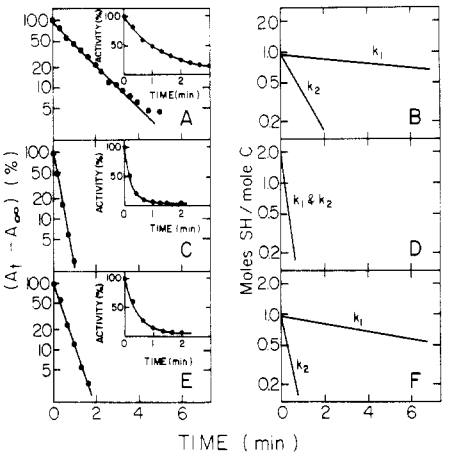


FIGURE 1: Effect of a protein and of a peptide substrate on the kinetics of inactivation of C by Nbs_2 and on the rate of modification of the enzyme sulfhydryl groups. (Panels A and B) No substrate added (reference experiments). The reaction mixtures (1.05 mL) contained C (4.8 μM) in a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0 (23 ± 1 °C). (Panels C and D) Experiments carried out in the presence of histone H2b (36 μM). The reaction mixtures (1.1 mL) contained C (4.4 μM) and histone H2b in the same Hepes–EDTA buffer. (Panels E and F) Experiments carried out in the presence of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (2 mM). The reaction mixtures (0.92 mL) contained C (3 μM) and the peptide in the same Hepes–EDTA buffer. The reaction was initiated for (A), (B), (C), and (D) by the addition of 50 μL of Nbs_2 (5 mM) in the above-mentioned Hepes–EDTA buffer and for (E) and (F) by the addition of 30 μL of the same Nbs_2 solution (to keep an approximate same excess of the thiol reagent over C). Panels A, C, and E show the first-order plots of the time courses of inactivation (cf. inserts), after correction for the residual activity of the fully modified enzyme, according to Ray & Koshland (1961). The activity of the enzyme was monitored by removing aliquots (25 μL) from the reaction mixtures, diluting them into 2.5 mL of an ice-cold buffer composed of Mes (0.1 M), pH 6.5, and immediately assaying 50 μL of these diluted solution. The activity was compared in each case to control systems that were identical in all respects except for the absence of Nbs_2. Panels B, D, and F show the first-order plots of the titrations of the SH groups in C.

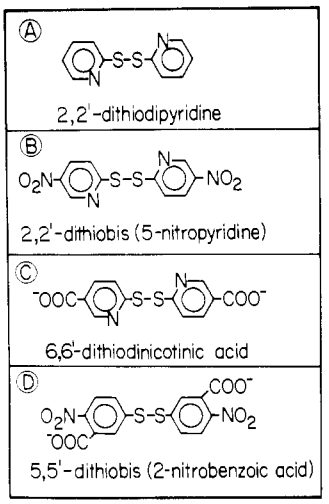
Table I: Effect of Peptide and Protein Substrates on the Rate Constants for the Inactivation of C by Nbs_2 and for the Modification of the Various Classes of Sulfhydryls in C by the Same Reagent[a]

substrate added	rate constants ($\times 10^{-3}$) (M^{-1} min^{-1})		
	k_1[c]	k_2[c]	k_{inact}[d]
none	0.2 ± 0.03	3.4 ± 0.5	3.0 ± 0.5
histone H2b	15.0 ± 2	15.0 ± 2	16.0 ± 2
protamine[b]	>20	>20	>20
Leu-Arg-Arg-Ala-Ser-Leu-Gly	0.3 ± 0.05	13.0 ± 1	12.0 ± 1

[a] Experimental conditions were as described in the legend to Figure 1. [b] The modification systems (0.9 mL) contained C subunit (3.2 μM) and protamine (80 μM) in a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0. The reaction was initiated by the addition of 30 μL of a solution of Nbs_2 (5 mM in the same buffer) and was followed as described in the legend to Figure 1. [c] Second-order rate constants of SH titration corresponding to the class I and II of thiols, respectively. [d] Second-order rate constants of inactivation.

Although these two proteins were found to accelerate the rate of chemical modification of both SH_I and SH_{II}, it seemed reasonable to assume that the accelerated inactivation of the enzyme results mainly from the increase in the reactivity of SH_{II} rather than SH_I, as we have previously shown that modification of SH_{II} alone suffices to inactivate the enzyme

Scheme I



(Jiménez et al., 1982). Furthermore, it could be argued that if the modification of either one of the two sulfhydryls by itself could cause a full inactivation of the enzyme, then the rate constant of inactivation (k_{inact}) should be equal to the sum of the rate constants for the modification of the two sulfhydryls ($k_1 + k_2$), i.e., $k_{inact} \simeq 3 \times 10^4$ M^{-1} min^{-1} (cf. Table I). However, the value of k_{intact} as determined experimentally in the presence of histone H2b was $(1.6 \pm 0.2) \times 10^4$ M^{-1} min^{-1}, very close to the rate of modification of one sulfhydryl alone.

The Bound Protein Substrate Channels the Negatively Charged Nbs_2 toward the SH Groups. Since both of the protein substrates used in these experiments (histone H2b and protamine) have a considerable net positive charge at neutral pH, we attempted to find out whether the enhanced reactivity of the sulfhydryls arises from the fact that the enzyme–substrate complex once formed simply attracts the negatively charged Nbs_2. We therefore used several disulfide thiol reagents (analogues of Nbs_2; Brocklehurst, 1979) which were either neutral or negatively charged (Scheme I) in order to determine whether there is a correlation between the negative charge of the reagent and the enhanced reactivity of the sulfhydryls in the enzyme–substrate complex. As seen in Figure 2, all these analogues inactivated the enzyme (though at different rates), and in all cases the inactivation could be quantitatively reversed by reaction with 2-mercaptoethanol to remove the modifying reagent, showing that the inactivation was due to the chemical modification itself and not to an irreversible denaturation triggered by the modification. Furthermore, in the presence of ATP the inactivation of the enzyme was attenuated, suggesting that it is associated with the modification of SH_{II} (cf. Jiménez et al., 1982).

When histone H2b is added to the reaction mixture and the inactivation of the enzyme is carried out in the presence of this protein substrate, it can readily be seen (Figure 2) that the rate of inactivation is greatly accelerated for the two reagents [6,6′-dithiodinicotinic acid, panel C; 4,4′-dithiobis-(3-nitrobenzoic acid), not illustrated] which like Nbs_2 (panel D) have a negative net charge under the conditions of the experiment. As for the thiol reagents which have no net charge under the same conditions [2,2′-dithiodipyridine, panel A; 2,2′-dithiobis(5-nitropyridine), panel B; 4,4′-dithiodipyridine, not illustrated] (Brocklehurst & Little, 1973; Grassetti & Murray, 1969), they inactivate the enzyme at a similar, if not slightly slower, rate in the presence of histone H2b.

Since the accelerating effect of histone H2b does not occur with all the Nbs_2 analogues but only with those having a

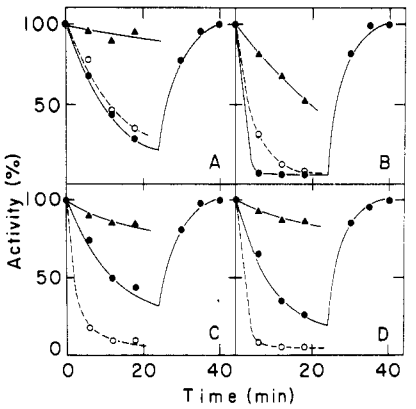


FIGURE 2: Correlation between the net charge of the thiol reagent used and the occurrence of an accelerated enzyme inactivation in the presence of histone H2b. All the reaction mixtures (0.6 mL) contained C (60 nM) in a buffer composed of Hepes (50 mM) and EDTA (0.5 mM), pH 7.5 (23 ± 1 °C), and in all cases the inactivation was initiated by addition of 50 μL of the indicated thiol reagent (0.15 mM in the same buffer). Reagents used: (panel A) 2,2′-dithiodipyridine; (panel B) 2,2′-dithiobis(5-nitropyridine); (panel C) 6,6′-dithiodinicotinic acid; (panel D) Nbs_2. With each of the various reagents used, the rate of inactivation of C alone (●) as well as the ability of histone H2b (final concentration 0.9 mg/mL) to accelerate the inactivation (○) and the protection from inactivation afforded by MgATP [final concentrations 0.2 mM ATP and 8.2 mM $Mg(CH_3COO)_2$] (▲) was monitored. The reactions were allowed to proceed for 24 min, and then the reversibility of the inactivation was checked in the reference system with C alone by testing the reactivation of the enzyme upon addition of 50 μL of 2-mercaptoethanol (1 M). The activity of the enzyme was monitored by assay of aliquots (50 μL) of the reaction mixtures at the indicated times, in comparison to control systems with buffer replacing the thiol reagent solution.

negative net charge, the major function of this protein substrate is not merely to promote an ionization of the thiols ($-SH \rightarrow -S^-$). Instead, it seems reasonable to assume that the enzyme–histone complex, once formed, somehow brings into play positively charged residues (unavailable in the free enzyme) that would channel the negatively charged reagent to the vicinity of the SH groups and increase its local concentration around its site of action. In principle, these positively charged residues could either be provided by the histone or become available in the enzyme itself around the sulfhydryls, as a result of a histone-induced conformational change.

The occurrence of such a histone-induced conformational change is quite likely in view of the finding (Jiménez et al., 1982; Kupfer et al., 1980) that the catalytic subunit of cAMP-dependent protein kinase has an intrinsically malleable structure and that it readily undergoes pronounced salt-induced conformational changes. On the other hand, both histone H2b and protamine are well-known as basic proteins and would therefore provide a considerable net positive charge to the enzyme–substrate complex.

It is interesting to note that the attenuation in the inactivation rate exerted by ATP occurs with all Nbs_2 analogues tested, regardless of their net charge, while the enhancement in the rate of inactivation caused by histone H2b or protamine is restricited to the negatively charged reagents only. This would be in line with the suggestion that ATP exerts its effect on the sulfhydryl (SH_{II}) itself (sterically shielding it or altering its state of ionization) while the protein substrates bring about their channeling effect by modulating the electric field in the microenvironment of the sulfhydryls. Furthermore, in a previous publication from this laboratory (Kupfer et al., 1979), it was shown that out of two alkylating reagents (N^{α}-tosyl-L-lysyl chloromethyl ketone, which has a net positive charge, and N^{α}-tosyl-L-phenylalanyl chloromethyl ketone, which is neutral), both of which inactivate the free C subunit by

modifying its SH_{II}, only the inactivation by the charged reagent is affected by the presence of histone H2b. In contrast to the channeling effect of histone H2b on negatively charged reagents, in this case the protein substrate protects the enzyme from inactivation by the positively charged reagent.

A Substrate Heptapeptide Enhances the Rate of Modification of SH_{II} Only. Since histone H2b and protamine are relatively large substrates with many positively charged amino acid residues, it is not possible to conclude from the fact that these proteins accelerate the rate of modification of both sulfhydryls that SH_I is necessarily vicinal to SH_{II} in the three-dimensional structure of the enzyme and thus that SH_I (like SH_{II}) is also located near the ATP binding site in C. Therefore, we attempted to restrict the accelerating effect of the protein substrate to the immediate vicinity of the ATP binding site by using a considerably smaller substrate (with only a few basic ammino acids) in order to find out whether the accelerated modification would then occur with both sulfhydryls or with one of them only.

For this purpose we used a heptapeptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly, which had been shown by Kemp et al. (1977) to constitute a good substrate for the enzyme having K_m and V_{max} values similar to those of histone H2b (Glass & Krebs, 1979; Jiménez et al., 1982). In addition, this heptapeptide contains only two basic amino acid residues at positions $n-2$ and $n-3$ from its target serine (n), which were shown to provide major recognition elements at the active site of the enzyme (Hjelmquist et al., 1974; Huang et al., 1974; Kemp et al., 1975; Zetterquist et al., 1976; Yeaman et al., 1976; Kemp et al., 1977).

As seen in Figure 1E, the binding of this heptapeptide (concentrations tested 0.3–2 mM) accelerates the rate of inactivation of C by Nbs_2 to almost the same extent as the binding of the much larger and more basic protein substrates (Table I). However, while in the case of histone H2b the kinetics of modification of the sulfhydryls is monophasic (i.e., both SH_I and SH_{II} are modified at a similar rate; Figure 1D), in the case of the heptapeptide the modification trace is biphasic (Figure 1F), and the acceleration in the rate of modification occurs essentially with SH_{II} only (Table I). As in the case of histone H2b, the net charge plays a crucial role: the sulfhydryl reagents with a negative net charge (Nbs_2 or 6,6′-dithiodinicotinic acid) inactivate the enzyme faster in the presence of the heptapeptide, while the rate of inactivation of the enzyme by the neutral reagent (2,2′-dithiodipyridine) is not affected by the presence of the heptapeptide (Figure 3). The above results suggest that SH_{II} is located in the vicinity of the active site, in agreement with our previous findings (Jiménez et al., 1982) which showed that SH_{II} is intimately associated with the γ-P subsite in the ATP binding site of the kinase. Furthermore, it can be inferred from the above that the channeling of the negatively charged reagents to the vicinity of SH_{II} may be attributed mainly to the positively charged amino acid residues which are considered essential in turning a given serine residue into a target for phosphorylation by this kinase. The fact that in the complex formed between C and its protein substrate the essential arginyl or lysyl residues of the substrate are close to SH_{II} is in line with our previous observation that N^{α}-tosyl-L-lysyl chloromethyl ketone (a chemically reactive derivative of lysine) acts as an affinity labeling reagent for C and inactivates this enzyme while reaching out with its chloromethyl ketone moiety to become covalently linked to SH_{II} (Kupfer et al., 1979).

Spatial Relationship between the Catalytic and Regulatory Subunits of the Kinase, As Reflected in the Reactivity of Their Sulfhydryls. It has been previously proposed that in the undissociated and inactive form of the kinase (R_2C_2), the R subunit shields the active site of the C subunit (Witt & Roskoski, 1975; Demaille et al., 1977; Hoppe et al., 1978; Kupfer et al., 1979). On the basis of the mapping of the ATP binding site in R_2C_2 (Hoppe et al., 1977) and in C (Hoppe et al., 1978), we came to the conclusion that while the shielding of the ATP site in C by R may be partial only, it most likely does involve the ribose triphosphate recognizing subsites in C (Hoppe et al., 1978). Therefore, if SH_{II} is located near the γ-P subsite of the ATP binding site of C (Jiménez et al., 1981), then one would expect that the reactivity of this sulfhydryl might be affected by the binding of R to C.

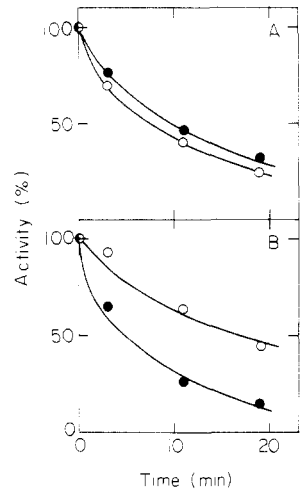


FIGURE 3: Correlation between the net charge of the thiol reagent used and the occurrence of an accelerated enzyme inactivation in the presence of the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly. The reaction mixtures (0.24 mL) contained C (0.4 μM) in a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0 (23 ± 1 °C), in the absence (○) or presence of Leu-Arg-Arg-Ala-Ser-Leu-Gly (0.5 mM) (●). Inactivation was initiated by addition of 20 μL of 2,2′-dithiodipyridine (0.2 mM) (panel A) and 6,6′-dithiodinicotinic acid (0.2 mM) (panel B). The activity of the enzyme was monitored by removing aliquots (25 μL) from the reaction mixtures, diluting them into 0.5 mL of an ice-cold Mes buffer (50 mM), pH 6.5, and immediately assaying 50 μL of these diluted solutions. The activity was compared in each case to control systems that were identical in all respects except for the absence of the thiol reagent.

As seen in Figure 4, this is indeed the case. Upon reaction with Nbs_2, the undissociated form of the enzyme (R_2C_2) loses its potential catalytic activity[2] at a much slower rate than its dissociated form [$R_2(cAMP)_4 + 2C$]. Fitting the time course of the inactivation to pseudo-first-order reaction kinetics yielded in both cases monophasic reaction traces (dashed lines in Figure 5). The rate constants for inactivation of the undissociated form of the enzyme ($k_{inact} = 15\ M^{-1}\ min^{-1}$) was over 130-fold slower than that of the dissociated form ($k_{inact} = 2000\ M^{-1}\ min^{-1}$; Table II).

The higher rate of inactivation of the dissociated form of the kinase cannot be simply attributed to the presence of an excess of cAMP, since it was shown by Armstrong & Kaiser (1978) that the rate of inactivation (by Nbs_2) of the C subunit alone is not affected by addition of cAMP and since the rate constant for inactivation of the dissociated enzyme ($k_{inact} = 2 \times 10^3\ M^{-1}\ min^{-1}$) is close to that of the free catalytic subunit ($k_{inact} = 3 \times 10^{-3}\ M^{-1}\ min^{-1}$; Jiménez et al., 1982).

It is interesting to compare the rate of inactivation by Nbs_2 of the type I kinase with that of the type II enzyme. As shown

[2] The undissociated form of this kinase (R_2C_2) is not catalytically active as such. Its potential catalytic activity is assayed following dissociation of the enzyme with cAMP.

Table II: Contribution of R (in R_2C_2) and of MgATP (Bound to Its High-Affinity Site) to the Protection of C from Inactivation by Nbs_2: Rate Constants of Inactivation and of SH Modification[a]

state of the enzyme prior to addition of Nbs_2					
catalytic activity	state of aggregation	nucleotide present	$10^{-3}k_{inact}$[b]	$10^{-3}k$[c]	moles of SH modified per mole of RC[d]
active	dissociated (R_2 + 2C)	cAMP		>10	1.2
			2.0	2.0	0.95
				0.17	1.1
				0.007	1.1
potentially active	undissociated (R_2C_2)	none		>7	1.0
				0.2	1.07
			0.015	0.018	3.3
potentially active	undissociated (R_2C_2) and "locked"[e]	MgATP		>10	1.0
				0.3	1.1
				0.03	2.0
			>0.001		

[a] The experimental conditions were as described in the legends to Figures 4 and 5. [b] Second-order rate constants of inactivation (M^{-1} min^{-1}). The potential catalytic activity of C in R_2C_2 was revealed, in each case, by including cAMP in the assay. [c] Second-order rate constants of modification of the various sulfhydryl groups in the enzyme (M^{-1} min^{-1}). [d] Values are given per RC (and not per R_2C_2) for convenience in comparison to the SH titrations of C alone (cf. Jiménez et al., 1982). [e] cf. the text.

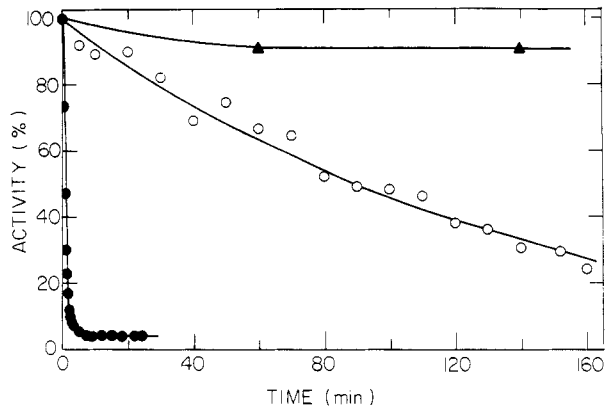


FIGURE 4: Protection of C from inactivation by Nbs_2 when found in the undissociated enzyme form (R_2C_2) and blockage of the inactivation of C by the binding of MgATP to its high affinity site in R_2C_2. The reaction mixture (0.65 mL) for monitoring the inactivation of R_2C_2 (○) contained R_2C_2 (2.3 μM) in a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0 (23 ± 1 °C). The reaction mixture for monitoring the inactivation of the dissociated enzyme (●) was identical except for the presence of cAMP (0.22 mM), and the reaction mixture (1.1 mL) used to establish the protection afforded by MgATP (▲) contained R_2C_2 (2.2 μM), ATP (11 μM), and $Mg(CH_3COO)_2$ (4.5 mM) in the same Hepes–EDTA buffer. The reaction was initiated by the addition of 45 μL of Nbs_2 (10 mM) for (○) and (●) and 75 μL of the same Nbs_2 solution for (▲). The phosphotransferase activity of C was determined by assays carried out in the presence of cAMP (5 μM). At the indicated times the residual activity of C was monitored as described in the legend to Figure 1.

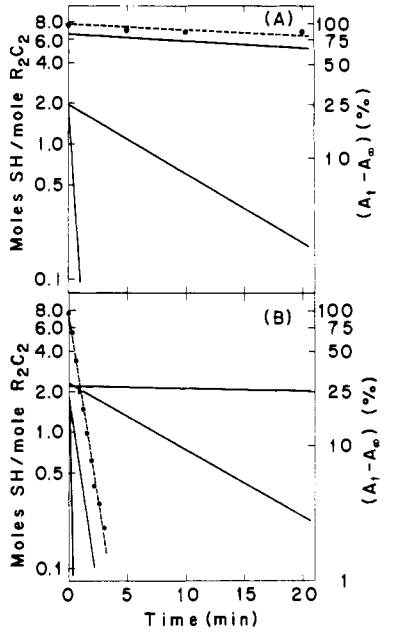


FIGURE 5: Comparison between the first-order kinetics of inactivation of C in R_2C_2 (---) and the modification of the various classes of sulfhydryls in R_2C_2 (—). (Panel A) Modification systems for titration of sulfhydryls (0.86 mL) contained R_2C_2 (2.3 μM) in a buffer composed of Hepes (0.1 M) and EDTA (1 mM), pH 7.0 (23 ± 1 °C). (Panel B) Modification systems were identical with those described in (A) except for the presence of cAMP (0.22 mM). The reactions were initiated by the addition of 60 μL of an Nbs_2 solution (10 mM in the same buffer) and was analyzed as described under Materials and Methods. The first-order plots of inactivation given were obtained from analysis of the data presented in Figure 4.

above, the rate of inactivation of the dissociated type I kinase (from rabbit muscle) is similar to, if not slower than, that of the pure C subunit. However, in the case of the type II kinase (from bovine heart), it was reported that the rate of inactivation in the presence of cAMP is considerably faster than that of the free subunit (Armstrong & Kaiser, 1978). This distinct difference between the two types of kinase is consistent with our hypothesis regarding the structural aspects of the interaction between the C subunit and its protein substrates, since one of the major differences between the two isozymes is that while the R subunit of type II is phosphorylated by C, the R subunit of type I is not (Rosen & Erlichman, 1975; Hofmann et al., 1975). Being a substrate for C, the type II regulatory subunit should contain structural features of a substrate, and indeed it has been proposed by Corbin et al. (1978) that arginyl residues are present in the vicinity of the serine residue which is phosphorylated in the type II R subunit.

More recently, Potter & Taylor (1979) sequenced the phosphorylated site in the type II R subunit (from porcine skeletal muscle) and found it to have the sequence Asx-Arg-Arg-Val-Ser(P)-Val, i.e., to contain the essential positively charged amino acid residues usually found in the substrates of this enzyme. These arginyl residues, which are present in the type II (but not in the type I) R subunit, may very well channel the negatively charged Nbs_2 to SH_{II} and thus accelerate the rate of its modification and the subsequent inactivation of the enzyme. In other words, the accelerated inactivation of C in the presence of the type II regulatory subunit could be due to the channeling effect created by the arginyl residues in the complex $R_2^{II}(cAMP)_4C_2$, which is analogous to the channeling

effect created by the arginines of the heptapeptide mentioned above, when bound to C.

The Type I Regulatory Subunit in R_2C_2 Shields SH_{II} and Protects C from Inactivation by Nbs_2. The large number of SH groups present in the R_2C_2 form of the enzyme (7 ± 0.3 mol of SH groups per mol of RC reacts with Nbs_2 in the presence of 2% sodium dodecyl sulfate) made it necessary to use a nonlinear regression computer program (Fletcher, 1971) for analyzing the kinetics of modification of these sulfhydryls by Nbs_2. It was found that the thiol groups in R_2C_2 displayed considerably different reactivities toward Nbs_2, and it was therefore possible to discriminate between distinct classes of sulfhydryls.

As seen in Figure 5B, fitting the time course of the reaction between the dissociated enzyme and Nbs_2 to a sum of exponents yields four linear reaction traces, which can be assigned to the modification of four classes of sulfhydryls. The number of modified sulfhydryls belonging to each class and their corresponding rate constants of modification are given in Table II. One of these classes of thiols, which corresponds to the titration of 0.95 mol SH per mol RC, is shown to be modified with a rate constant of 2×10^3 M^{-1} min^{-1} (Table II), i.e., close to the rate constant of modification of SH_{II} in pure C (3×10^3 M^{-1} min^{-1}; cf Table I). Interestingly, the modification of this class of sulfhydryls was found to occur concomitantly with the loss of potential catalytic activity of the enzyme (Figure 5B and Table II), which would strongly support the identification of this sulfhydryl group as the previously described essential SH_{II} in C.

When R_2C_2 is allowed to react with Nbs_2 in the absence of cAMP, the kinetics of the reaction can be fitted to only three classes of thiol groups. Most strikingly, the class of thiols that in dissociated R_2C_2 had a reactivity similar to that of SH_{II} (rate constant 2×10^3 M^{-1}) disappears (cf. Figure 5). Instead, the class of thiols which had the lowest reactivity (18 M^{-1} min^{-1}) is composed now of 3 mol of SH groups per mol of RC, compared with only 1.1 mol in the undissociated enzyme. The fact that the loss of potential catalytic activity of R_2C_2 occurs (in the absence of cAMP) concomitantly with the modification of these least reactive sulfhydryls (Figure 5A and Table II) suggests that one of these three SH groups may well be the SH_{II} of C, whose modification was shown to bring about inactivation of the enzyme. Presumably, the reactivity of SH_{II} is attenuated in R_2C_2 due to shielding by R. This suggestion is further supported by our previous observation (Kupfer et al., 1979) that R (type I) shields the SH_{II} of C from modification by N^{α}-tosyl-L-lysyl chloromethyl ketone and prevents the inactivation of C by this reagent.

Comparing the stoichiometry of modification of the dissociated and undissociated forms of R_2C_2 clearly shows that the undissociated form has one additional sulfhydryl per RC, which is not available in the dissociated enzyme. This additional SH group reacts with Nbs_2 with a rate constant of 18 M^{-1} min^{-1}. Since it disappears upon dissociation of the enzyme by cAMP, it is reasonable to assume that it might originate from the R subunit and that it becomes shielded by the binding of cAMP to R. This possibility is supported by the finding of Armstrong & Kaiser (1978) that cAMP blocks the titration of one SH group in purified R (type II).

It should also be noted that in both the dissociated and undissociated forms of R_2C_2 there is one class of thiols that reacts with Nbs_2 with a rate of 170–200 M^{-1} min^{-1} (Table II), which is identical with the rate of modification of SH_I in C (rate constant 200 M^{-1} min^{-1}). If this resemblance is not fortuitous, then it suggests that the reactivity of SH_I is not affected by the binding of R to C.

Spatial Relationship between SH_{II} and the High-Affinity ATP Site in R_2C_2. The undissociated form of R_2C_2 (type I) is known to possess a high-affinity site for ATP (K_d = 50 nM; Haddox et al., 1972; Beavo et al., 1975). It has been previously proposed that this high-affinity site for ATP in R_2C_2 partially overlaps the ATP site in C (Hoppe et al., 1978), with the regulatory subunit probably shielding the ribose triphosphate moiety in C. Since ATP attenuates the Nbs_2 inactivation of C by shielding SH_{II} (Jiménez et al., 1982), one could expect that locking the high-affinity ATP site in R_2C_2 with ATP would further attenuate this inactivation of C. As seen in Figure 4, this is indeed the case; when R_2C_2 is exposed to Nbs_2 for a prolonged period (up to 5 h) in the presence of MgATP (ATP, 11 μM; $Mg(CH_3COO)_2$, 4.5 mM), the undissociated enzyme still retains >85% of its original potential activity, though 4.1 mol of SH groups per RC are modified after 5 h (not illustrated). For comparison, when R_2C_2 is allowed to react for 5 h with Nbs_2 in the absence of ATP, the undissociated enzyme loses essentially all its potential catalytic activity, and 5.4 mol of SH groups per RC becomes modified.

It seems therefore that upon addition of ATP to R_2C_2 its C subunit becomes considerably more resistant to inactivation by Nbs_2. This resistance is expressed in the dramatic attenuation in the rate at which R_2C_2 loses its potential catalytic activity and in the fact that the total number of SH groups that can be titrated drops in the presence of ATP by 1.3 SH groups per RC. It should be noted that (a) the concentration of ATP which is sufficient for full protection of R_2C_2 is quite low (3 μM), corresponding to the high affinity of R_2C_2 for ATP, and ~50-fold lower than the concentration needed to afford protection to pure C and (b) the rate of inactivation of C (even at very high ATP concentration, 1 mM) is >100-fold faster than the rate of loss of potential catalytic activity of R_2C_2 in the presence of much lower ATP concentrations (3 μM). It can therefore be concluded that ATP exerts it protective effect in R_2C_2 by binding to its high-affinity site and that it locks the enzyme in its R_2C_2 form, shielding further the SH_{II} group in C and making it inaccessible to attack by Nbs_2.

Conclusion

The results presented in this paper suggest not only that SH_{II} is structurally associated with the γ-P subsite of the ATP binding site in C (Jiménez et al., 1982) but also that in the complex formed between the enzyme and its protein substrates this sulfhydryl group resides in the vicinity of one or more of the essential arginyl or lysyl residues in the substrate, which were previously shown to constitute major recognition elements at the active site of the enzyme (Hjelmquist et al., 1974; Huang et al., 1974; Kemp et al., 1975; Zetterquist et al., 1976; Yeaman et al., 1976; Kemp et al., 1977; Kupfer et al., 1979).

Since a large protein substrate (histone H2b) considerably accelerates the rate of modification of both SH_I and SH_{II}, while a small heptapeptide substrate restricts the acceleration to SH_{II} only, it becomes possible to use a series of peptide substrates containing the sequence of the heptapeptide mentioned above and additional basic amino acid residues at an increasing distance from the target serine and to determine at which stage the channeling effect extends to include SH_I. Such studies would shed light on the spatial relationship between the two sulfhydryls.

Finally, the channeling effect of the heptapeptide substrate provides an example of how the specificity of a chemical modification can be sharpened by a preferential (substrate-mediated) targeting of the reagent to a desired functional

group in the enzyme. In a previous publication (Jiménez et al., 1982) we have shown that it is possible to change the relative rate of modification of SH_{II} and SH_I from a ratio of 1:1 to 16:1 by slightly modulating the ionic strength of the medium. By the additional resolution achieved here through the use of the heptapeptide, the relative rate of modification of the two sulfhydryls can be made to reach a ratio of 40:1. In other words, it becomes possible to essentially complete the modification of SH_{II} before SH_I starts to be modified (cf. Jiménez et al., 1982). Furthermore, since Nbs_2 is a fully reversible label, it can also be used to temporarily mask SH_{II}, while SH_I is irreversibly tagged with an appropriate reagent, thus obtaining an enzyme exclusively labeled at SH_1. Once confirmed by sequence studies, this selective labeling approach may prove valuable for establishing structure–function relationships in this enzyme.

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