SUBSTRATE-MEDIATED CHANNELING OF A CHEMICAL REAGENT TO THE ACTIVE SITE OF cAMP-DEPENDENT PROTEIN KINASE

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1. Introduction

The specific affinity between enzymes and their physiological ligands (substrates, coenzymes) has been widely used for achieving selective labeling of enzyme active sites. This approach, most commonly referred to as 'affinity labeling' [1] has given rise to several variations and refinements, e.g., the introduction of transition-state analogs and mechanism-based irreversible inhibitors. In affinity labeling reagents, the biorecognition elements (which target the reagent to the active site) and the chemically reactive group (which anchors the reagent at the active site) are both part of the same molecule. We describe here a case in which a carefully designed peptide substrate is used to preferentially channel a separate group-specific reagent to one (kinetically characterized) sulfhydryl group at the active site of cAMP-dependent protein kinase.

2. Materials and methods

The following materials were prepared according to described procedures: free catalytic subunit of cAMPdPK [2]; histone H2b [3]; the peptide Leu–Arg–Arg–Ala–Ser–Leu–Gly [4].

Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; cAMPdPK, cAMP-dependent protein kinase; C, catalytic subunit of cAMPdPK; Nbs_2, 5,5′-dithiobis [2-nitrobenzoic acid]

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[γ-^{32}P]ATP (2.0–2.7 Ci/mmol) was obtained from the Radiochemical Centre, Amersham; Nbs_2, 2,2′-dithiodipyridine, 2,2′-dithiobis[5-nitropyridine], 6,6′-dithiodinicotinic acid and 4,4′-dithiodipyridine were obtained from Sigma. 4,4′-dithiobis (3-nitrobenzoic acid) was a gift of Professor M. Fridkin. All other chemicals were best available grade from commercial sources.

The assay of cAMPdPK was carried out as in [5]. The enzyme preparations used had spec. act. 8–13 units/mg. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the transfer of 1 μmol ^{32}P from [γ-^{32}P]ATP onto histone H2b/min at pH 6.5 and 30°C.

Protein concentrations were determined by the method in [6]. M_r = 40 000 was used for the C subunit of cAMPdPK [7] and M_r = 13 800 for histone H2b [8]. The chemical modification of the SH groups of the enzyme was monitored and analyzed as in [9,10].

3. Results and discussion

Recent sequence studies have shown that the catalytic subunit (C) of cAMPdPK contains 2 Cys residues [11]. When the SH groups of these residues are allowed to react with Nbs_2 at neutral pH and an ionic strength of 0.22, the enzyme is inactivated, both the inactivation and the SH modification taking place with monophasic kinetics (fig.1, cf. also [9]). However, if the reaction is done at an ionic strength of 0.03, two clear-cut phases can be distinguished in the modification process: a slower phase (rate constant I, fig.1B) corresponding to the titration of 0.9 ± 1.0 mol/SH groups/mol C and attributed to

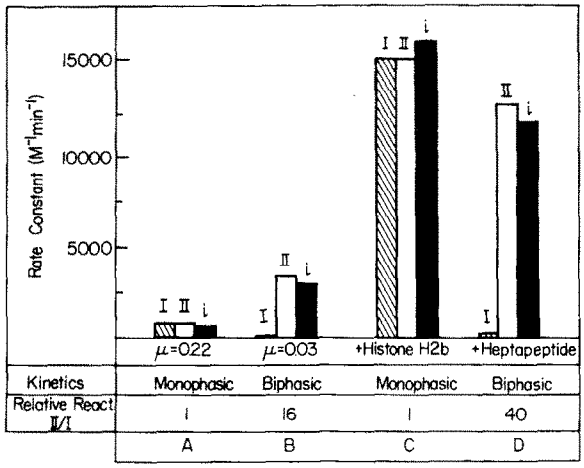

Fig.1. Effect of ionic strength, of histone H2b and of the heptapeptide (Leu–Arg–Arg–Ala–Ser–Leu–Gly) on the rate constants of modification of SH_I (denoted I) and SH_{II} (denoted II) by Nbs_2, as well as on the rate of inactivation of the enzyme (denoted 'i') by this reagent. The modification was carried out at pH 7.0 and 23°C with a ~50-fold molar excess of Nbs_2 over the enzyme (4.8 μM), as in [9]. Note that the modification of the SH groups is monophasic in the presence of histone H2b (36 μM) but biphasic in the presence of the heptapeptide (2 mM).

SH_I [9], and a faster phase (rate constant II, fig.1B) corresponding to the titration of 1.0 ± 0.1 mol SH groups/mol C and attributed to SH_{II}. Under these conditions, the inactivation of the enzyme (rate constant 'i', fig.1B) occurs concomitantly and stoichiometrically with the modification of SH_{II} [9], suggesting that this SH group might be located at the active site of the enzyme. In addition, the rate of inactivation of the enzyme and the rate of modification of SH_{II} were found to be dramatically attenuated in the presence of MgATP, the nucleotide substrate of the kinase. In the absence of the γ-P (as in MgADP) the protection afforded by the nucleotide substrate analog was considerably lowered, and in the absence of both the γ-P and β-P (as in MgAMP) there was no protection from inactivation. On the basis of these results it was concluded that SH_{II} is at least structurally associated with the γ-P subsite of the ATP binding site in C, if it does not constitute an integral part of it.

Since the catalytic function of cAMPdPK is to transfer the γ-P from ATP onto a target serine residue of the protein substrate, it seemed obvious that the subsites accomodating these two substrates are vicinal in the 3-dimensional structure of the enzyme, and therefore that the presence of a protein substrate at its site might affect the chemical reactivity of SH_{II} and consequently the inactivation of the enzyme by Nbs_2. Upon testing this assumption it was found that in contrast to MgATP (which dramatically attenuates the rate of inactivation of the enzyme by Nbs_2) the protein substrate histone H2b brings about an opposite effect, considerably accelerating both this inactivation, and the rate of chemical modification of the enzyme (fig.1,2). However, unlike MgATP (which exerts its protective effect on SH_{II} only), histone H2b accelerates the rate of modification of the two

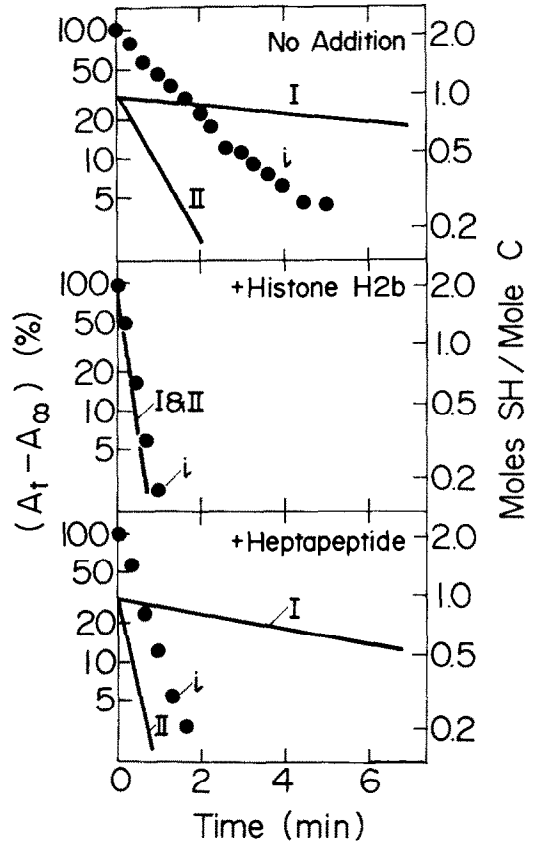

Fig.2. Effect of histone H2b and of the heptapeptide (Leu–Arg–Arg–Ala–Ser–Leu–Gly) on the kinetics of inactivation of C (•) by Nbs_2 and on the rate of modification of SH_I (denoted I) and of SH_{II} (denoted (II)). The reaction was done at pH 7.0 and 23°C with a ~50-fold molar excess of Nbs_2 over the enzyme (4.8 μM) and the kinetics was measured as in [9]: upper, reference experiment with no substrate added; center, experiment done in the presence of histone H2b (36 μM); lower, experiment done in the presence of the heptapeptide (2 mM).

sulfhydryls in C, making the kinetics of the reaction monophasic (fig.2).

As we have shown that modification of SH_{II} alone suffices to inactivate the kinase [9], it was reasonable to assume that the accelerated inactivation of the enzyme in the presence of histone H2b might be due to the increase in the reactivity of SH_{II} rather than SH_I. Furthermore, it could be argued that if the modification of either one of the two SH groups could cause a full inactivation of the enzyme, then the rate constant of inactivation (i) should be equal to the sum of the rate constants for modification of the two sulfhydryls (I + II). In other words, the rate constant in the presence of histone H2b should be equal to $\sim 3 \times 10^{-4}$ $M^{-1} . min^{-1}$ while under these conditions 'i' was determined to be $\sim 1.6 \times 10^4$ $M^{-1} . min^{-1}$ (fig.1C), very close to the rate constant for the modification of one SH only.

In view of the fact that histone H2b is a basic protein with a considerable net charge at neutral pH (where the Nbs_2-modification of the enzyme is carried out), we attempted to find out whether the enhanced rate of modification observed in the presence of this protein does not arise simply from the fact that upon binding of this histone to the enzyme, the complex formed attracts the negatively charged Nbs_2 reagent and leads or 'channels' it to react with the SH groups of the enzyme (histone H2b has no SH groups that would react by themselves with this reagent). This working hypothesis was supported by the finding that the rate of inactivation of the enzyme in the presence of histone H2b is accelerated when negatively-charged Nbs_2 analogs are used, but not when they are replaced by neutral analogs of this thiol reagent (fig.3).

On the basis of these findings we tried to restrict the accelerating effect of histone H2b, which was exerted on both SH_I and SH_{II} (making the kinetics of modification monophasic, fig.1C,2), and to limit it to the immediate vicinity of the ATP-binding site, i.e., to SH_{II} only. Such local preferential acceleration of the modification of SH_{II} was achieved by replacing the large histone H2b substrate with a much smaller substrate, the peptide Leu–Arg–Arg–Ala–Ser–Leu–Gly, which had been shown [4] to constitute a good substrate for cAMPdPK, with K_m- and V_{max}-values similar to those determined with histone H2b [9,12].

As seen in fig.1D, this heptapeptide accelerates the rate of inactivation of C by Nbs_2 to almost the same

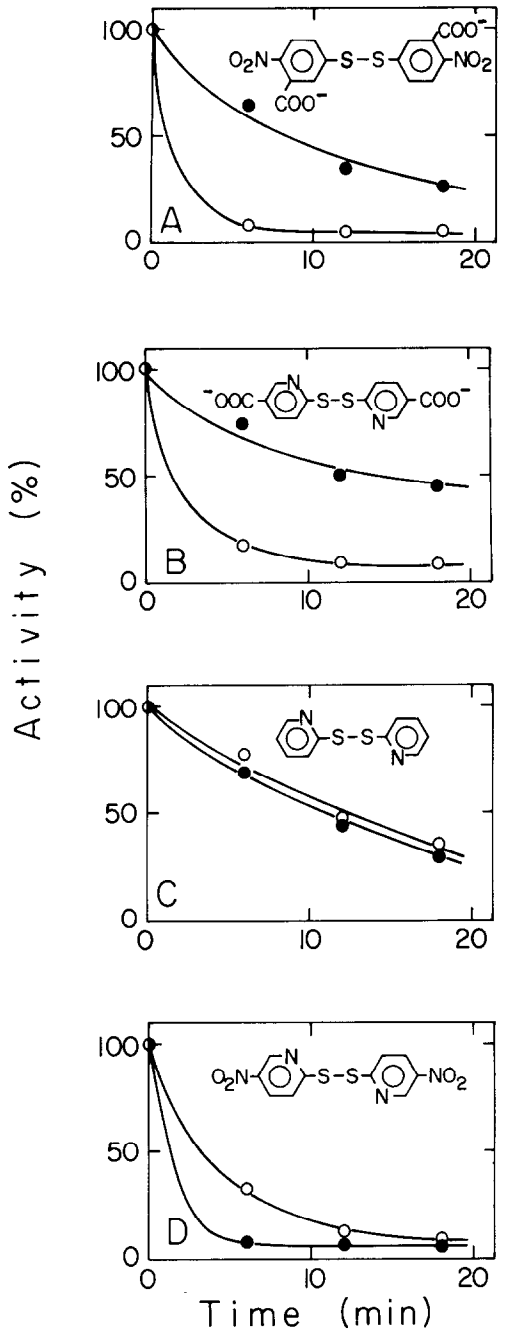

Fig.3. Correlation between the net charge of the Nbs_2 analog and the occurrence of an accelerated enzyme inactivation in the presence of histone H2b. The inactivation was allowed to proceed at pH 7.5 (23°C) and with ~150-fold molar excess of Nbs_2 over the enzyme (60 nM). With each of the reagents used, the rate of inactivation of C alone (•) as well as the ability of histone H2b (○; final conc. 0.9 mg/ml) to affect the inactivation were monitored.

extent as histone H2b (cf. fig.1C,D). However, while in the case of histone H2b the kinetics of modification of the sulfhydryls is monophasic (both SH_I and SH_{II} are modified at the same rate (fig.2)) in the case of the heptapeptide the modification of the SH groups in the enzyme is clearly biphasic, and the acceleration in the rate of modification occurs practically with SH_{II} only (fig.2). This preferential and restricted acceleration of SH_{II} is better reflected in the rate constants depicted in fig.1. Upon comparing the experiments 'C' and 'D' (fig.1) it is evident that the relative reactivity of SH_{II} to SH_I increases from 1 in the presence of histone H2b to 40 in the presence of the heptapeptide. The fact that the relative rate of modification of the two sulfhydryl groups in the enzyme can be made to reach such a high ratio (40:1) sets the stage for achieving a selective preferential labeling of SH_{II}, since with such relative rates it becomes possible to essentially complete the modification of SH_{II} before SH_I starts to be modified.

Furthermore, since the label introduced upon reacting SH groups with Nbs_2 can be quantitatively removed with thiols under very mild conditions, the results presented above may also be used for the selective labeling of SH_I. For example, it becomes possible to temporarily mask SH_{II} with Nbs_2, label SH_I with an alkylating reagent (such as ICH_2COOH, a chloromethylketone [5]) and then unmask SH_{II} with 2-mercaptoethanol.

The above results can be accounted for if the spatial relationship between SH_{II}, SH_I and the active site of the enzyme (which accomodates ATP and the protein substrate) is assumed to be as schematically depicted in fig.4.

According to this scheme, SH_{II} is located at the active site and more specifically, it is close to the subsite accomodating the γ-P of ATP. Also, in the complex formed between the enzyme and its protein substrates, SH_{II} is vicinal to the essential arginyl or lysyl residues of such substrates [13], the positively charged residues adjacent to the serine which becomes phosphorylated during the catalytic event. Placing SH_{II} in this position could account for a large number of observations reported here, as well as in other publications from our laboratory. These include:

(i) MgATP protects SH_{II} from reaction with Nbs_2;

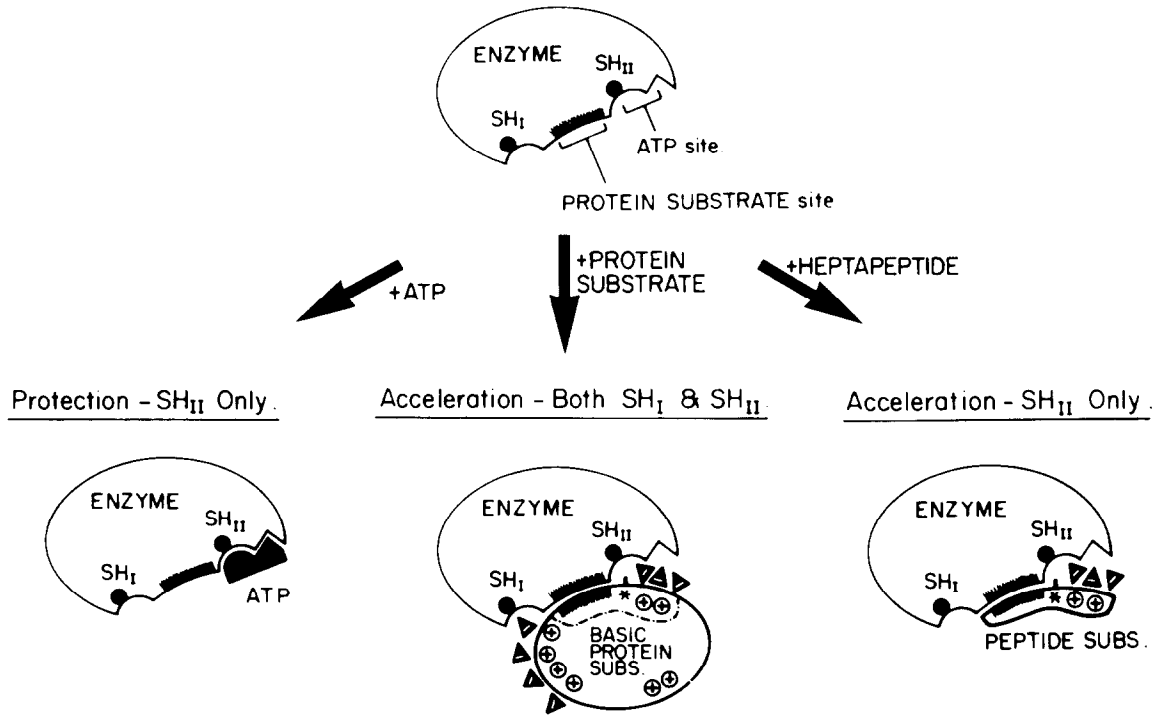

Fig.4. Schematic representation of the spatial relationship between SH_{II}, SH_I and the sites which accommodate ATP and the protein (or peptide) substrates of the enzyme.

(ii) MgATP attenuates dramatically the inactivation of the enzyme by Nbs_2, concomitantly and stoichiometrically with the above protection;

(iii) MgADP is much less effective in that respect and MgAMP fails altogether to afford protection both to SH_{II} (from reaction with Nbs_2) and to the enzyme (from inactivation);

(iv) Protein substrates such as histone H2b (or protamine) enhance the rate of inactivation of the enzyme by Nbs_2 and concomitantly the rate of modification of SH_{II} (but also of SH_I);

(v) A similar accelerated inactivation can be achieved by the heptapeptide Leu–Arg–Arg–Ala–Ser–Leu–Gly but in this case the enhanced rate of modification is restricted to SH_{II} only;

(vi) For both (iv) and (v) the accelerated inactivation and modification is achieved with negatively charged analogs of Nbs_2 but not with similar analogs devoid of negative charge;

(vii) N^{α}-tosyl-L-lysine–chloromethylketone (an affinity labeling reagent for lysine-recognizing sites in proteins) inactivates the enzyme with concomitant binding to SH_{II} [5]. This inactivation does not take place (under the same conditions) with $ClCH_2COOH$, and equally reactive reagent devoid of the structural elements of lysine;

(viii) MgATP protects the enzyme from inactivation by this chloromethylketone [5].

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 enzyme. Furthermore, we would have to assume that protein substrates such as histone H2b and protamine, and even the heptapeptide mentioned above, are capable of imposing a conformational change that would considerably enhance the reactivity of this distal SH_{II}. We would also have to assume that this enhanced reactivity would be restricted to negatively charged (but not to neutral) analogs of Nbs_2. We would also have to assume a coincidental occurrence of a lysine recognizing site also at the vicinity of this distal SH_{II}.

While we cannot, at this stage, completely dismiss the occurence of such a combination of possibilities, it seems that placing SH_{II} at the active site itself (fig.4) accounts for all the above observations and, what is more, it is a simpler and more plausible assumption.

In any case, the labeling approach illustrated here, which is based on the combined use of a small, carefully chosen substrate together with a separate group-specific reagent, is useful in and of itself, since it enables us to optimalize the specificity of tagging of a unique site in the enzyme. The SH_{II} group which is labeled here certainly resides in a location which is conformationally linked to the active site, if it does not actually reside at the active site proper [14,15]. Therefore the labeling of this particular sulfhydryl is most useful in the preparation of derivatives of cAMPdPK for X-ray crystallography and for further structure–function studies.

Generally speaking, the use of a substrate for channeling an appropriate companion-reagent to the active site of an enzyme has the following advantages:

1. Since the chemically reactive moiety and the bio-recognition functional groups do not reside on the same molecule, the interference of undesirable side-reactions (e.g., ring closure) is minimized, both during synthesis and during the labeling reaction itself;
2. When the affinity between the targeting moiety and the enzyme is not very high, it is possible to ensure saturation of the active site by increasing the concentration of the substrate only, without having to increase at the same time the concentration of the chemically reactive species. This favours active-site specificity by lowering the probability of attack at other loci of the enzyme.

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