AFFINITY LABELING OF RABBIT SKELETAL MUSCLE PHOSPHORYLASE KINASE BY 5′-(*p*-FLUOROSULFONYLBENZOYL)ADENOSINE

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1. Introduction

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) is a regulatory enzyme in the cascade of reactions associated with glycogenolysis. The enzyme from white skeletal muscle of the rabbit is a hexadecamer of 4 dissimilar subunits, $\alpha_4\beta_4\gamma_4\delta_4$ [1], whose activity is markedly stimulated by μM levels of Ca^{2+} [2–4]. Although the γ subunit has catalytic activity [5,6] and the δ subunit is identical to calmodulin [7], the roles of the two largest subunits, α and β, have not been clearly defined. Modification of both the β and γ subunits was observed [8] employing a series of alkylating ATP affinity labels, but inactivation was found to be correlated with modification of the β subunit. We have also sought to exploit affinity labeling by ATP analogs as a method for identification of possible catalytic and regulatory sites on individual subunits as they occur in the native enzyme. We have found that μM levels of the photolabel 8-azido ATP cause preferential labeling of the β subunit of phosphorylase kinase [9]. The 2′,3′-dialdehyde derivative of ATP was also found to be an effective affinity label at very low concentrations, but it did not form an isolatable derivative which would have allowed identification of modified subunits [10]. Here, we extend these studies on the interaction of phosphorylase kinase with ATP affinity labels by reporting on its interaction with 5′-(*p*-fluorosulfonylbenzoyl)adenosine (5′-FSBA). Its use revealed the existence of a binding site on phosphorylase kinase with a high affinity for free ADP. In the absence of Mg^{2+} this nucleotide exclusively protected the β subunit against modification by 5′-FSBA.

2. Materials and methods

Purification of phosphorylase kinase and phosphorylase *b*, the assay for phosphorylase kinase activity, and the procedure for gel electrophoresis were done as in [10]. An M_r of 335 000 for each tetramer ($\alpha\beta\gamma\delta$) of phosphorylase kinase was utilized for calculating the stoichiometry of labeling [11]. For fig.2 and table 1 phosphorylase kinase concentrations were determined following [12].

5′-FSBA was synthesized as in [13] and was recrystallized from dimethylformamide. For the synthesis of 5′-[2-^{3}H]FSBA the reaction utilized 5 mCi [2-^{3}H]adenosine and was scaled down to 1/20th. 5′-[2-^{3}H]FSBA was purified over Silica gel-60 [14] and was used at spec. act. 2.4×10^{13} dpm/mol. [γ-^{32}P]ATP was prepared as in [15].

3. Results and discussion

Incubation of phosphorylase kinase at pH 8.2 and 30°C with mM levels of 5′-FSBA resulted in the rapid loss of enzymatic activity (fig.1); incubation of the kinase in the absence of the analog, but under otherwise identical conditions, had very little effect. When inactivation of phosphorylase kinase was carried out with increasing [5′-FSBA], saturation kinetics were observed. This data is plotted in the inset of fig.1 as $E_o/(E_o-E)$ *vs* the reciprocal of [5′-FSBA], where E_o is the activity of enzyme incubated in the absence of inhibitor, and E is the activity of enzyme incubated with 5′-FSBA. From the intercept on the axis of the abscissa an app. K_i of 0.8 mM was obtained for 5′-FSBA.

A 2.5-fold increase in the rate of inactivation was caused by the synergistic action of Mg^{2+} and Ca^{2+} at

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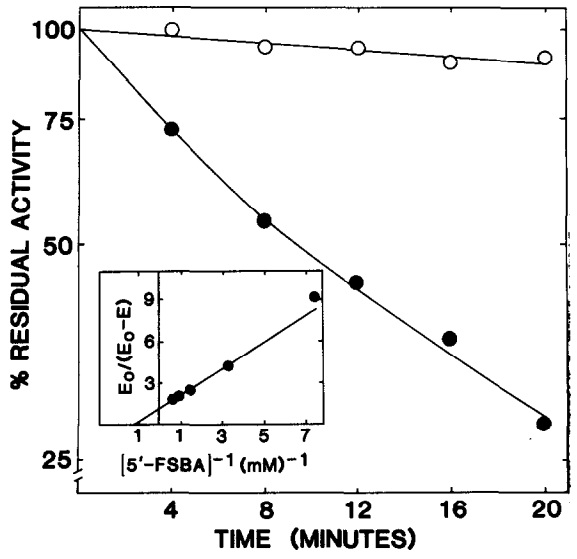

Fig.1. Inactivation by 5'-FSBA. Non-activated phosphorylase kinase (0.2 mg/ml) was inactivated at 30°C by 5'-FSBA (1.0 mM) in the presence of Hepes buffer (30 mM, pH 8.2), dimethylformamide (4%, v/v) and EDTA (0.2 mM). At the indicated times aliquots were initially diluted 60-fold with cold buffer (40 mM Hepes, 30 mM mercaptoethanol, pH 6.8) and then diluted 6-fold into an assay mixture containing in final conc.: kinase (0.55 μg/ml); phosphorylase (7.8 mg/ml); MgATP (10 mM $Mg(CH_3CO_2)_2$, 1.5 mM [^{32}P]ATP at 15.5 Ci/mol); $CaCl_2$ (0.2 mM); EDTA (0.1 mM); mercaptoethanol (12 mM); and buffer (92 mM Hepes, pH 8.0). After 3 min at 30°C aliquots were removed for the determination of ^{32}P incorporation into protein: (○) incubation without 5'-FSBA; (●) incubation with 5'-FSBA. Inset: Determination of an app. K_i for 5'-FSBA. Inactivation of phosphorylase kinase was carried out with increasing [5'-FSBA] (up to 1.6 mM). Incubation and assay conditions were as above except that dimethylformamide was 5% in the inactivation mixture and [^{32}P]ATP was 2.0 mM at 6.8 Ci/mol in the assay mixture.

10 mM and ~0.1 mM, respectively. Separately, a <50% increase in the rate of inactivation was observed when Mg^{2+} alone were added to the modification reaction, whereas no change in the inactivation rate was caused by Ca^{2+} in the absence of Mg^{2+}. In [10] we observed a synergistic effect of Ca^{2+} on the inactivation of phosphorylase kinase by an affinity label of ATP with the 2',3'-dialdehyde derivative of ATP. This suggests that the 2 ATP-analogs may inactivate phosphorylase kinase at a common site.

Modification of phosphorylase kinase by 5'-[^{3}H]-FSBA resulted in the incorporation of radioactive analog into the α,β and γ subunits; however, labeling of the β subunit was 2–3-times greater. Incorporation of ^{3}H into the δ subunit was not determined because the protein band corresponding to this subunit was too diffuse under our electrophoresis conditions to ensure a quantitative recovery of protein. In order to determine which of the 3 subunit-modifications was responsible for the loss of enzymatic activity, inactivation by 5'-FSBA and incorporation of the analog into the individual subunits were determined simultaneously (table 1). The results showed, however, that inactivation of phosphorylase kinase by 5'-FSBA can-

Table 1
Inactivation and subunit labeling by 5'-FSBA

Inactivation time (min)	Inactivation (%)	Subunit-labeling (mol 5'-FSBA/mol subunit)		
		α	β	γ
5	33.6 ± 3.5	0.25 ± 0.09	0.54 ± 0.04	0.26 ± 0.05
10	56.1 ± 2.1	0.45 ± 0.08	1.00 ± 0.24	0.41 ± 0.07
15	66.3 ± 0.8	0.76 ± 0.13	1.66 ± 0.20	0.68 ± 0.09

Non-activated phosphorylase kinase (0.44 mg/ml) was inactivated by 5'-[2-^{3}H]FSBA (0.8 mM) at 30°C in the presence of EDTA (0.2 mM), dimethylformamide (4%, v/v), and buffer (50 mM Hepes, pH 8.2). At the indicated times aliquots of the reaction were stopped by addition of a small aliquot of dithiothreitol (final conc. 100 mM). Residual activity was determined as in fig.1. In addition, aliquots of each sample were filtered over Sephadex G-50 [16] followed by SDS gel electrophoresis to determine the incorporation of ^{3}H into the individual subunits. The values shown are averages with standard deviations of results obtained in triplicate

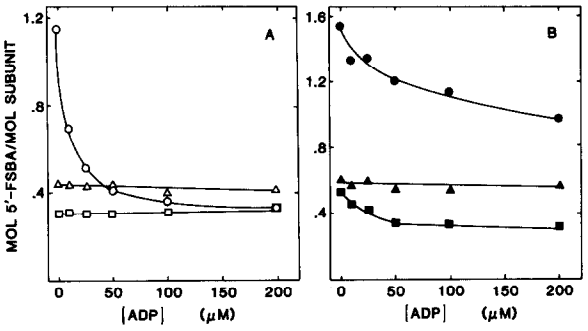

Fig.2. Effect of ADP on subunit-labeling by 5′-FSBA. Non-activated phosphorylase kinase (0.8 mg/ml) was inactivated by 5′-[2-^{3}H]FSBA (0.5 mM) in the presence of EGTA (0.2 mM), dimethylformamide (4%, v/v), Hepes buffer (50 mM, pH 8.2), and the indicated [ADP]. After 15 min at 30°C the reactions were terminated by addition of a small aliquot of dithiothreitol (final conc. 100 mM). The modified enzyme was separated from excess reagent by filtration over Sephadex G-50 [16] and then SDS gel electrophoresed for determination of ^{3}H incorporation into the individual subunits: (A) minus Mg^{2+}; (B) plus 10 mM $Mg(CH_3CO_2)_2$ in the modification; (△,▲) α; (○,●) β; (□,■) γ.

not be directly correlated with labeling of any particular subunit, because incorporation of the analog into each of the 3 subunits was sufficient to account for the loss of enzymatic activity.

To determine the specificity of modification, we tested the ability of AMP, ADP and ATP to protect individual subunits from being modified. The extent to which a nucleotide blocks labeling in a given time, along with the concentration of nucleotide necessary to achieve the protection, can indicate the specificity of the protected site for the affinity label. The most dramatic results were obtained with ADP. In the absence of Mg^{2+}, this nucleotide was found to exclusively protect the β subunit from labeling by 5′-FSBA, and the concentration of ADP necessary for this protection was surprisingly low (fig.2A). A decrease of >60% in labeling of the β subunit by 0.5 mM 5′-FSBA was obtained in the presence of 50 μM ADP, at which point the effect of ADP reached a plateau. Under the conditions in fig.2A, 8.8 μM ADP was sufficient to obtain the half-maximal effect, which indicates that free ADP binds rather tightly to the enzyme.

Fig.2B shows the effect of ADP on subunit-labeling in the presence of 10 mM Mg^{2+}, which was considerably different than that observed in the absence of this ion. Under these conditions labeling of the γ subunit, as well as labeling of the β subunit, was significantly reduced in the presence of ADP. For the γ subunit this effect of ADP in the presence of Mg^{2+} reached a plateau at ~50 μM ADP. The protective effect on the β subunit did not reach a plateau even at 200 μM ADP.

In separate experiments the effects of 0.2 mM AMP and 0.2 mM ATP on subunit labeling by 5′-FSBA were determined. When tested under the conditions in fig.2, this concentration of AMP had no effect on labeling of any of the subunits. The same concentration of ATP was found to protect all 3 subunits to approximately the same extent; however, the protection by ATP was significant only in the presence of Mg^{2+}.

Because ADP was effective in protecting phosphorylase kinase from affinity labeling by 5′-FSBA we determined its effect on the inactivation caused by this analog. Table 2 shows the results of such an experiment in which inactivation of the enzyme by 5′-FSBA was done in the absence or presence of ADP or MgADP. Despite the protection by ADP against labeling of the β subunit, it offered relatively little protection against inactivation by 5′-FSBA. Nevertheless, protection by ADP against inactivation again reached a plateau at <50 μM. The fact that both labeling of the β subunit and inactivation of phosphorylase kinase by 5′-FSBA were reduced in the presence of free ADP, but that this protective effect reached a plateau, could be explained by a model in which 5′-FSBA and ADP bind to functionally distinct sites on the enzyme. In such a model saturation by ADP at one site would cause a decreased rate of affinity labeling by 5′-FSBA at a distinct site. Alternatively,

Table 2
Effect of ADP on inactivation by 5′-FSBA

Additions	% Control activity
5′-FSBA	50.4 ± 1.4
5′-FSBA + ADP (0.2 mM)	66.5 ± 1.1
5′-FSBA + Mg^{2+} (10 mM)	24.9 ± 4.4
5′-FSBA + Mg^{2+} (10 mM) + ADP (0.2 mM)	60.4 ± 4.1

Inactivation of phosphorylase kinase by 0.5 mM 5′-FSBA and assays for enzymatic activity were done as in fig.1 except that $Mg(CH_3CO_2)_2$ and/or ADP were added to the inactivation mixture where indicated. The activity of phosphorylase kinase incubated in the absence of 5′-FSBA, but in the presence of the respective effectors, represented 100% of control activity. The values shown are averages with standard deviations of results obtained in triplicate

similar results would be obtained if there were 2 separate binding sites for 5′-FSBA on the β subunit, and if ADP competed predominantly at only one of the sites.

Addition of Mg^{2+} only slightly influenced the protection afforded by ADP against inactivation by 5′-FSBA (table 2). Protection from inactivation by various concentrations of MgADP, when tested under the conditions and concentrations described for fig.2B, did not show the plateau that it had with free ADP (not shown). Even though there was a large excess of free Mg^{2+} in these experiments, a known effector of phosphorylase kinase, the differences in the protection from inactivation by ADP plus and minus Mg^{2+} was due to the effect of MgADP rather than free Mg^{2+}. This is clearly seen in table 2, where it is shown that free Mg^{2+} enhances, rather than protects against, the inactivation by 5′-FSBA.

5′-FSBA is an affinity label of phosphorylase kinase which preferentially labels the β subunit. Protection from labeling by this analog was obtained in the presence of μM levels of ADP, which suggests that a high affinity binding site for free nucleotide exists on the enzyme. It is not yet possible to deduce the function or location of this binding site; affinity labeling with an ADP analog of greater affinity and specificity than that obtained with 5′-FSBA may serve to answer these questions.

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