# Selective labelling of phosphorylase kinase with fluorescein isothiocyanate

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Fluorescein isothiocyanate (FITC) is a highly specific inhibitor of rabbit muscle phosphorylase kinase. The rapid inhibition process is accompanied by an almost exclusive incorporation of fluorescein into the α subunit. A molar ratio of 0.8 mol FITC per mol α subunit for a 60% inhibited kinase was calculated. $Mg^{2+}$ and $Mg^{2+}$-ATP completely block the inhibitory effect of FITC, but ATP, ADP and $Ca^{2+}$ have no significant effect on FITC inhibition. Trypsin-activated phosphorylase kinase is not inactivated by FITC, while the fluorescein-modified enzyme can be activated by digestion with trypsin to the same level of activity of trypsin-activated unmodified enzyme.

*Phosphorylase kinase* *Fluorescent dye* *Chemical labelling* *Enzyme inactivation*

## 1. INTRODUCTION

Phosphorylase kinase from rabbit fast skeletal muscle is a multisubunit enzyme with a composition $\alpha_4\beta_4\gamma_4\delta_4$ [1]. The $\alpha$ subunit is replaced by $\alpha'$ in the isoenzyme from slow red skeletal muscle [2]. The enzyme phosphorylates glycogen phosphorylase and synthase [3,4], while it is also known to phosphorylate various proteins including troponins, H1 histone, $\kappa$-casein, myosin light chain and myelin basic protein (review [5,6]). Recent reports clearly show that the $\gamma$ subunit has catalytic activity and represents a protein which shows homology to the catalytic subunit of the cyclic AMP-dependent protein kinase [7,8]. However, there is no general agreement if phosphorylation of all protein substrates is carried out by a single type of catalytic site, and the role of the enzyme's two large subunits, $\alpha$ and $\beta$, has not been clearly defined. In this respect, $\beta$ subunit might also contain a catalytic center [9,10], while the N-terminal sequence of the $\alpha$ subunit shows homology to the $pp60^{v-src}$ tyrosine kinase [8].

*Abbreviation:* FITC, fluorescein 5-isothiocyanate

FITC, generally used as a nonspecific fluorescent probe for labelling of proteins, was demonstrated to be an efficient inhibitor of $Na^+,K^+$-ATPase and $Ca^{2+}$-ATPase of sarcoplasmic reticulum, while it has been suggested to selectively modify ATP binding sites of both ATPases [11–13]. In addition, two other fluorescein derivatives, tetraiodofluorescein and eosin, have been shown to act as 'adenosine analogues' and to bind effectively to a variety of nucleotide-binding enzymes [14–16].

In an effort to identify possible catalytic and/or regulatory sites on individual subunits of phosphorylase kinase, we found that FITC is an effective inhibitor of phosphorylase kinase and a specific fluorescent label for its $\alpha$ subunit.

## 2. MATERIALS AND METHODS

FITC, isomer I, was obtained from Serva. Trypsin (type 1) and soybean trypsin inhibitor were products of Sigma. [$\gamma$-$^{32}$P]ATP (3 Ci/mmol) was purchased from the Radiochemical Centre, Amersham. Crystalline rabbit skeletal muscle phosphorylase *b* was prepared as in [17]. Rabbit

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skeletal muscle phosphorylase kinase was purified as in [18]. Three different kinase preparations were used in this work with specific activities of approx. 5.1 units/mg at pH 8.6 and a pH 6.8/8.2 activity ratio of about 0.14, suggesting proteolytic activation [18].

Phosphorylase kinase activity was determined according to the method described in [18], which is based on the measurement of phosphorylase activity, with the following modifications. Phosphorylase *b* to *a* conversion was terminated by dilution in ice-cold 30 mM triethanolamine–HCl buffer, pH 6.8, containing 0.5 mg/ml bovine serum albumin, 1 mM EDTA and 20 mM 2-mercaptoethanol, and assayed for phosphorylase *a* as in [19] with 75 mM glucose-1-P. The unit of phosphorylase kinase activity was defined as in [18]. Phosphorylase kinase was alternatively assayed by measuring the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP into phosphorylase *b*. The incubation mixture was similar to that described in [1].

FITC-phosphorylase kinase conjugate was prepared by incubating the kinase (2 mg/ml) with FITC (10 $\mu$M) in 25 mM Tris–HCl, 0.1 mM EDTA buffer, pH 8.6, at 22°C. The reaction was terminated by separation of the unbound dye, using microcolumns with Sephadex G-50 previously swollen in the pH 8.6 buffer, according to the centrifuge column procedure [20]. The amount of FITC bound to the kinase was calculated from the absorption spectrum of the labelled enzyme, assuming an absorption coefficient at 502 nm of 80000, for fluorescein bound to protein [21]. Protein determinations for measuring stoichiometry of labelling were performed as in [22], while $M_r$ values of 305000 per tetramer ($\alpha\beta\gamma\delta$) and 132000 per $\alpha$ subunit were utilized [8]. Absorption and fluorescence measurements were performed on a Perkin-Elmer 356 spectrophotometer and a Perkin-Elmer 650-40 spectrofluorimeter, respectively.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 0.75 mm thick slab gels and a continuous buffer system, as described in [18]. The gels were stained with Coomassie brilliant blue. The fluorescent bands in unstained gels were visualized by illumination in a dark room with a long-wavelength UV lamp and photographed using a camera fitted with an orange filter.

## 3. RESULTS

Incubation of phosphorylase kinase (2 mg/ml) with a 6.1-fold molar excess of FITC, at pH 8.6, resulted in a rapid inactivation of the kinase along the time course shown in fig.1. 60% of the enzyme activity was lost during the first 3 min of incubation, while the total inactivation was incomplete and did not exceed 70%, even at 30 min of reaction. When the kinase was incubated with FITC for 3 min, as in fig.1, but in presence of $Mg^{2+}$-ATP (4 mM $Mg^{2+}$, 1.2 mM ATP), no inhibition was observed. Higher concentrations of FITC (up to 200 $\mu$M) incubated with phosphorylase kinase for 10 min, under the same conditions of fig.1, were not able to reduce the enzyme activity more than 75%.

Following the effect of FITC on the kinase activity at 10-fold lower enzyme concentration, it was observed that the dye/kinase molar ratio needed for effective inhibition was significantly higher (fig.2). In this case, 50% inhibition was obtained, when phosphorylase kinase was incubated with 5 $\mu$M FITC for 10 min. The presence of 5 mM

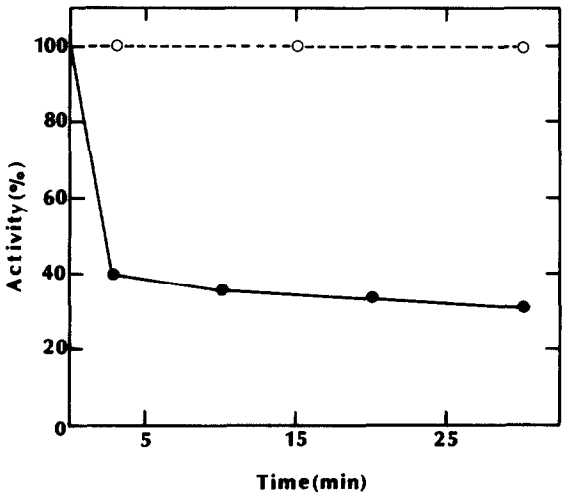


Fig.1. Time course of inhibition of phosphorylase kinase activity by FITC. Phosphorylase kinase (2 mg/ml) was incubated at pH 8.6 (25 mM Tris–HCl, 0.1 mM EDTA) in presence (●—●) or absence (○---○) of 10 $\mu$M FITC, for different times at 22°C. The incubation was terminated by 100-fold dilution in ice-cold pH 6.8 buffer (50 mM glycerol-2-P, 20 mM 2-mercaptoethanol) and the kinase activity was assayed by phosphorylase activation at pH 8.6.

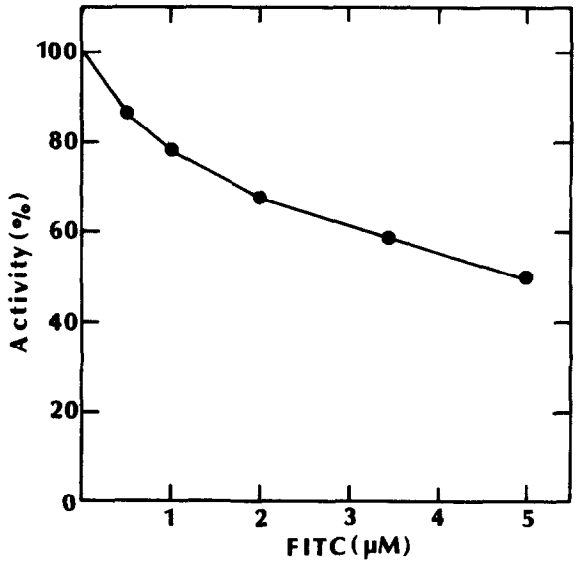


Fig.2. Inactivation of phosphorylase kinase by different FITC concentrations. Phosphorylase kinase (0.2 mg/ml) was incubated at pH 8.6 (25 mM Tris–HCl, 0.1 mM EDTA) with various FITC concentrations, for 10 min at 22°C. The reaction was terminated by 10-fold dilution and the kinase activity assayed as in fig.1.

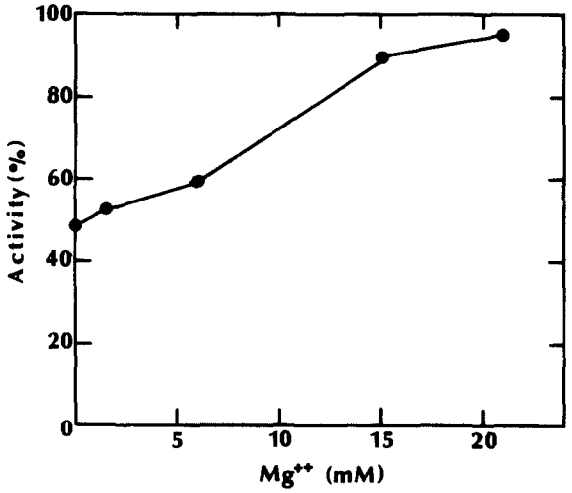


Fig.3. Effect of $Mg^{2+}$ on the inactivation of phosphorylase kinase by FITC. Phosphorylase kinase (0.2 mg/ml) was incubated at pH 8.6 (25 mM Tris–HCl, 0.1 mM EDTA) with 5 $\mu$M FITC for 10 min at 22°C, in presence of increasing concentrations of $Mg(CH_3COO)_2$. The reaction was terminated by 10-fold dilution and the kinase activity assayed as in fig.1. 100% is the activity of unlabelled phosphorylase kinase, while the activity of the enzyme modified with FITC in absence of $Mg^{2+}$ is 48% that of native kinase.

ATP or ADP or 1 mM $Ca^{2+}$ in the incubation mixture of fig.2 (5 $\mu$M FITC) did not protect against FITC inhibition. In contrast, the presence of 20 mM $Mg^{2+}$, during the incubation of the kinase with the fluorescent dye, was sufficient to offer almost complete protection against enzyme inhibition. Lower $Mg^{2+}$ concentrations were found less effective (fig.3).

SDS–gel electrophoresis of the FITC-enzyme conjugate, prepared using the reaction conditions of fig.1 and a short reaction time, revealed that the inhibition process was accompanied by an almost exclusive incorporation of fluorescein into the $\alpha$ (and $\alpha'$) subunit (fig.4). No significant fluorescence was observed in the positions of $\gamma$ and $\delta$ subunits (examined also with 7.5% gel). Only two very faint fluorescent bands were sometimes seen, one in the position of $\beta$ subunit and the other in the position of a 75-kDa protein band (usually present in the kinase preparations), which is probably a proteolytic fragment of $\alpha$ or $\beta$ subunits [3,18].

The absorption maximum of the FITC-kinase conjugate was shifted from 494 nm (free FITC) to 502 nm, which is typical for protein-bound fluorescein [23], while the fluorescence emission maximum of the conjugate occurs at 524 nm. The stoichiometry of labelling for the above conjugate was calculated to be about 0.8 mol fluorescein per mol $\alpha$ subunit of the 60% inhibited kinase.

To investigate further the role of $\alpha$ subunit in the inactivation of phosphorylase kinase by FITC, we have examined the action of trypsin on the activity of the kinase-FITC system, at pH 8.6, before and after modification with the fluorescein derivative. As is known, trypsinolysis is accompanied by a marked degradation of the $\alpha$ and $\beta$ subunits and an activation of the kinase, especially evident at pH 6.8 [3,18]. Table 1 shows that trypsin-activated phosphorylase kinase is not inactivated by FITC, while, as shown in table 2, the FITC-modified enzyme can be activated by proteolysis to about the same level of activity of trypsin-activated unmodified enzyme.

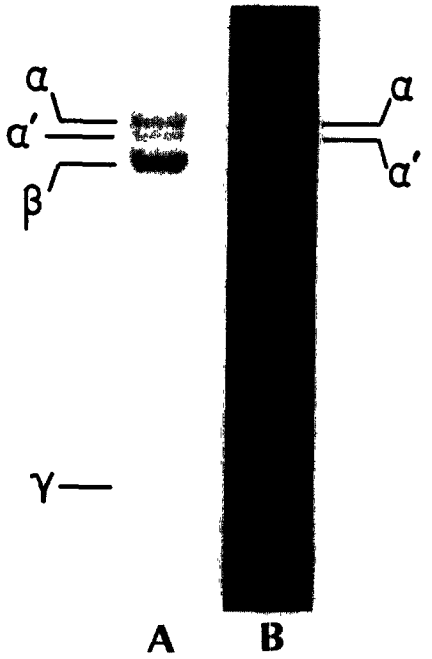


Fig.4. SDS–PAGE of FITC-phosphorylase kinase conjugate. 70 μg of FITC-phosphorylase kinase conjugate, prepared as described in section 2 and denatured as in [18], were applied to 5% slab gel. A, Coomassie blue-stained gel; B, fluorescence of the unstained gel. Enlargement of B is slightly greater than that of A.

Table 1

Effect of FITC on the activity of native and trypsin-activated phosphorylase kinase

| Enzyme form | Activity (%) at pH 6.8 |
|---|---|
| Phosphorylase kinase (native) | 100 |
| Phosphorylase kinase + FITC | 55 |
| Phosphorylase kinase (trypsinized) | 312 |
| Phosphorylase kinase (trypsinized) + FITC | 369 |

Phosphorylase kinase (2 mg/ml) was incubated for 40 min, in presence or absence of trypsin (4.5 μg/ml), in 2 mM EDTA, 50 mM glycerol-2-P buffer, pH 6.8. Proteolysis was terminated by a 10-fold dilution with 25 mM Tris–HCl, 0.1 mM EDTA buffer, pH 8.6, containing 22 μg/ml trypsin inhibitor and the reaction with FITC (10 μM) started immediately. After 10 min, the modification reaction was terminated by a 10-fold dilution in the pH 6.8 buffer containing 0.5 mg/ml bovine serum albumin. Phosphorylase kinase (3 μg/ml) was assayed at pH 6.8 using the radioactive assay

Table 2

Effect of trypsin on the activity of native and FITC-modified phosphorylase kinase

| Enzyme form | Activity (%) at pH 6.8 |
|---|---|
| Phosphorylase kinase (native) | 100 |
| Phosphorylase kinase + FITC | 45 |
| Phosphorylase kinase + trypsin | 345 |
| (Phosphorylase kinase-FITC) + trypsin | 320 |

Phosphorylase kinase (2 mg/ml) was incubated in presence or absence of 10 μM FITC as in fig.1. The reaction was terminated after 3 min by 5-fold dilution with the pH 6.8 buffer of table 1, containing in addition 20 mM 2-mercaptoethanol. Activation of the kinase (0.4 mg/ml) by trypsin (2 μg/ml) was carried out for 15 min in the pH 6.8 buffer. Proteolysis was terminated by a 20-fold dilution with ice-cold pH 6.8 buffer containing 20 μg/ml of trypsin inhibitor and 0.5 mg/ml bovine serum albumin and the kinase was assayed as in table 1

## 4. DISCUSSION

FITC appears to act as a selective label for the α (and α') subunit of phosphorylase kinase, and is a very potent inhibitor of the phosphorylase *b* to *a* conversion. One possible explanation of the incompleteness of inactivation process may be a partial proteolytic degradation of the kinase preparations. This hypothesis is also strengthened by the results of proteolysis experiments (tables 1,2). The inability of ATP or ADP to protect the kinase against FITC inhibition was somewhat puzzling, as it is already known that this fluorescein derivative is a selective affinity probe for the ATP binding site of certain enzymes [11–13]. However, the protective effect of $Mg^{2+}$-ATP and $Mg^{2+}$ (fig.3) permits the assumption that the fluorescein derivative modifies a $Mg^{2+}$-ATP binding site of a catalytic domain in the α subunit, and that the $Mg^{2+}$-ATP complex reacts on the site of $Mg^{2+}$ with the enzyme molecule [23]. The above results are in accordance with a previous report which indicated that α subunit can be photoaffinity-labelled with 8-azido-ATP, suggesting that this subunit may not be excluded from posssessing a catalytic domain [10]. It remains of course a possibility, that FITC modifies

a regulatory site of $\alpha$ subunit and that the protective effect of free $Mg^{2+}$ is due to a direct action of this metal ion on the enzyme conformation.

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