Volume 268, number 1, 133–136 FEBS 08685 July 1990

3,4-Dichloroisocoumarin, a serine protease inhibitor, inactivates glycogen phosphorylase *b*

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Received 8 May 1990; revised version received 4 June 1990

3,4-Dichloroisocoumarin (3,4-DCI) is a highly reactive, mechanism-based inhibitor of serine proteases. We show here that glycogen phosphorylase *b* is also inactivated by this inhibitor, in a mechanism that parallels the inactivation of serine proteases, but involving multiple sites of covalent modification. Such a process may compromise studies in which 3,4-DCI is used to arrest proteolysis of a second native protein which may itself be modified.

3,4-Dichloroisocoumarin; Glycogen phosphorylase *b*

1. INTRODUCTION

Limited proteolysis of native enzymes often requires rapid and irreversible inactivation of the protease for subsequent analysis of the products of the digestion. Several strategies are available, including denaturation of the reaction mixture, use of an immobilised protease, removal of the protease by an immobilised inhibitor or addition of a reversible, and preferably irreversible inhibitor [1].

3,4-Dichloroisocoumarin (3,4-DCI) was designed as a general mechanism-based serine protease inhibitor capable of inactivating most elastase-like, chymotrypsin-like and trypsin-like proteases [2]. Inactivation involves the acylation of an active site serine by 3,4-DCI, concurrent with isocoumarin ring opening [3] (Fig. 1). 3,4-DCI has several attractive features: it is an easily handled crystalline material, is stable for months in organic solvents such as DMSO, is reasonably stable in aqueous media, is generally much more reactive towards serine proteases than phenylmethanesulphonylfluoride and is far less toxic than di-isopropylphosphofluoridate. Thus, 3,4-DCI is of value in the prevention of undesirable proteolysis due to serine proteases [2] and the characterisation of the role of serine proteases in biological processes [3].

However, the usefulness of 3,4-DCI in such roles depends critically upon 3,4-DCI possessing high activity towards serine proteases but very low activity towards other enzymes and cellular constituents. It has been reported that 3,4-DCI does not react with the thiol protease papain, the metalloprotease leucine aminopeptidase or β-lactamase [2]. For studies on limited proteolysis of glycogen phosphorylase *b* in vitro, the need for rapid inactivation of the protease, and instability of 3,4-DCI in aqueous media meant that it was necessary to use relatively high concentrations of the inhibitor. Under such conditions, we have noted that glycogen phosphorylase *b* is itself susceptible to inactivation by 3,4-DCI.

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Abbreviations: 3,4-DCL, 3,4-dichloroisocoumarin; DMSO, dimethylsulphoxide

2. MATERIALS AND METHODS

2.1. *Materials*

We purchased the following chemicals from the sources indicated: pure rabbit skeletal muscle glycogen phosphorylase *b* (University of Dundee); 3,4-dichloroisocoumarin, adenosine-5′-monophosphate (AMP), glucose-1-phosphate (BCL, Lewes, East Sussex); glycogen type II from oyster, ammonium metavanadate, pyrophosphate (Sigma Chemical Co., Poole, Dorset). All other chemicals were of analytical grade.

2.2. *Glycogen phosphorylase* b *assay*

Phosphorylase *b* activity was assayed in the direction of glycogen synthesis and inorganic phosphate release using a modification of the method of Carney et al. [4]. The sample to be assayed was first diluted using 0.1 M maleate buffer, pH 6.8. The diluted sample (10 μl) was added to 10 μl buffered substrate (0.15 M glucose-1-phosphate, 2.0 mM AMP, 2% (w/v) glycogen, 0.1 M maleate, pH 6.8) in a 1.5 ml microcentrifuge tube previously equilibrated to 30°C. After incubation for 15 min at 30°C, 0.98 ml colour reagent (2 mM ammonium metavanadate, 0.57 mM ammonium molybdate, 0.14 M HCl; [5]) was added. The absorbance of the reaction mixtures at 310 nm was measured against a colour reagent blank. The assay is linear to 50 nkat (nmol/s) phosphorylase *b*.

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00145793/90/$3.50

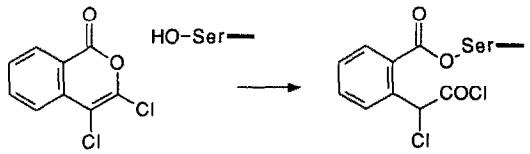

Fig. 1. Reaction of 3,4-DCI with serine proteases (after [3]).

2.3. *Inactivation of phosphorylase* b *by 3,4-DCI*

Phosphorylase *b* was diluted from a glycerol stock (100 mg/ml) to 0.5 mg/ml in 50 mM pyrophosphate buffer, pH 7.4. 3,4-DCI was added from a DMSO stock (50 mM) such that the final concentration was between 100 and 500 μM. At times thereafter, samples were removed and assayed for residual phosphorylase *b* activity. Control incubations included enzyme in the presence or absence of solvent. In other experiments, the absorbance of the reaction mixture was monitored at 325 nm to monitor the ring opening due to 3,4-DCI reaction. The stoichiometry of the modification was determined using a molar extinction coefficient of 3670 $M^{-1} \cdot cm^{-1}$ after correction for the non-specific hydrolysis of 3,4-DCI. In other incubations, samples were removed and resolved by anion-exchange chromatography.

2.4. *Anion-exchange chromatography*

Samples of phosphorylase *b*, and phosphorylase *b* inactivated with 3,4-DCI were filtered through a 0.22 μm filter before application to a 1.0 ml Mono-Q column previously equilibrated in 10 mM pyrophosphate buffer, pH 7.4. A linear gradient of 0–1 M NaCl in the same buffer was used to elute phosphorylase *b* and modified species. The eluate was monitored at 280 nm.

3. RESULTS AND DISCUSSION

Phosphorylase *b* activity is completely stable for 120 min under the conditions described, in both the absence and presence of DMSO. However, addition of 3,4-DCI leads to rapid inactivation of the enzyme. The reaction is dependent on the concentration of 3,4-DCI but cannot be analysed as a simple first-order process (Fig. 2). When simple exponential decay curves were fitted by non-linear regression [6], the sign and magnitude of the residuals correlated with progress through the reaction, implying that the inactivation might be considered as a sum of several exponential events, consistent with multiple reaction sites. 3,4-DCI-inactivated phosphorylase *b* was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis under which conditions it ran as a single protein band (M_r = 97.4 kDa) even after complete loss of activity (results not shown).

It was likely that 3,4-DCI was acting as an inactivator/inhibitor of phosphorylase *b*, a reaction that could involve acylation/ring opening by the same mechanism as for inhibition of serine proteases. To test this, we monitored the long wavelength absorption peak at 325 nm which is associated with the isocoumarin ring system (Fig. 3). Upon ring opening, the absorbance at 325 nm is reduced to negligible levels during serine protease inactivation [2]. The change in absorption at 325 nm can also be used to define the

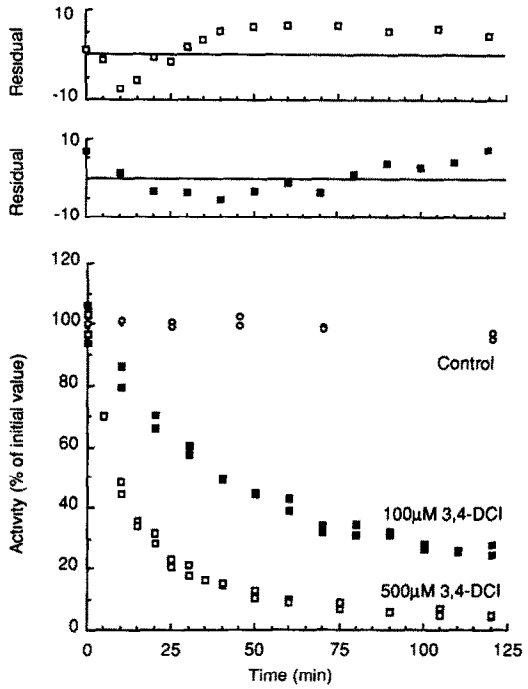

Fig. 2. Inactivation of phosphorylase *b* by 3,4-DCI. Glycogen phosphorylase *b* (0.5 mg/ml) was incubated in 50 mM pyrophosphate buffer, pH 7.4, in the presence of 100 μM (■), 500 μM (□) 3,4-DCI or in the presence of solvent alone (5% (v/v) DMSO; ○). At suitable times samples were removed and assayed for residual glycogen phosphorylase *b* activity. Data are presented as individual values from duplicate experiments. Simple first-order exponential equations ($A_t = A_0 \exp(-kt)$) were fitted to the data and the residuals for the inactivation reactions for 100 μM (■) and 500 μM (□) 3,4-DCI are shown (inset).

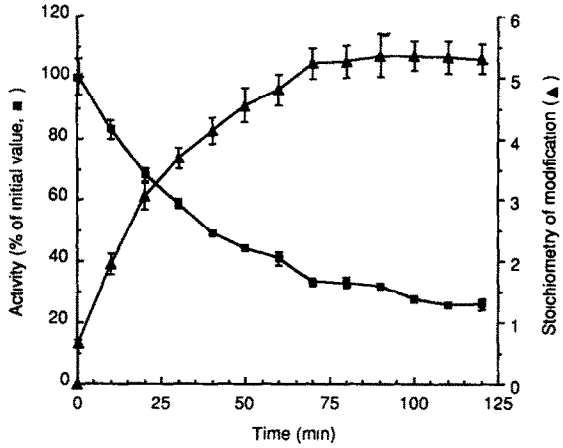

Fig. 3. Stoichiometry of modification of phosphorylase *b* by 3,4-DCI. Glycogen phosphorylase *b* (0.5 mg/ml) was incubated in 50 mM pyrophosphate buffer, pH 7.4, in the presence of 100 μM (■) 3,4-DCI or in the presence of solvent alone. The rate of inactivation of phosphorylase *b* was assayed as described previously. At the same time, a parallel incubation was monitored at 325 nm to measure ring-opening of 3,4-DCI to derive the stoichiometry of modification of phosphorylase *b* (▲). This value was corrected for the non-specific hydrolysis of inhibitor during the reaction by comparison with a reagent blank. Data are presented as mean ± range for a duplicate set of experiments.

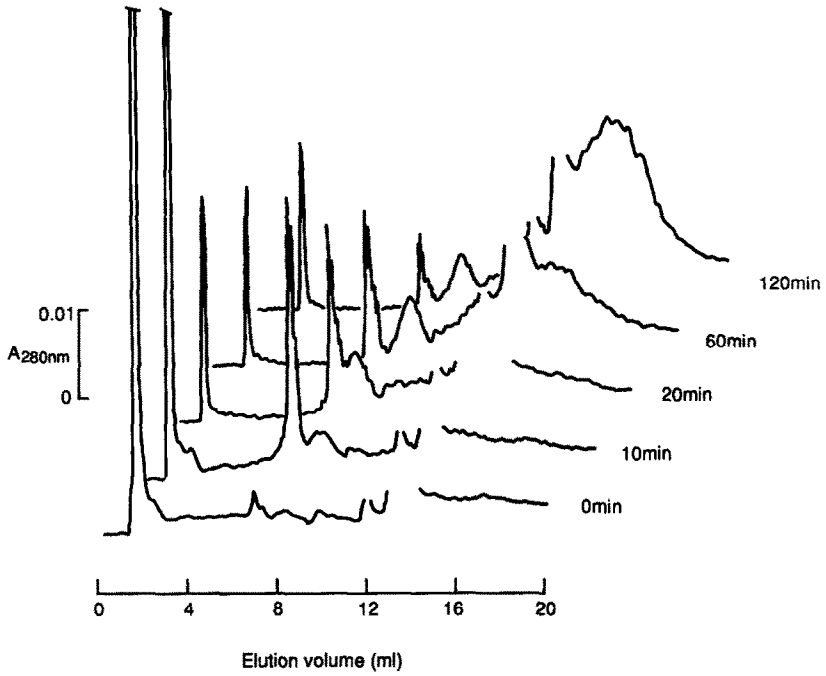

Fig. 4. Covalent modification of phosphorylase *b* by 3,4-DCI. Glycogen phosphorylase *b* (0.5 mg/ml) was incubated with 500 μM 3,4-DCI. At suitable times, samples were removed and separated by chromatography at 1.0 ml/min on Mono-Q anion-exchange media. The gradient (not shown for clarity) was as follows: 0–2.5 min: 0% B; 2.5–7.5 min: 0–100% B linear; 7.5–9.5 min: 100% B; 9.5–10 min: 100–0% B linear. Buffer A was 10 mM pyrophosphate buffer, pH 7.4, buffer B was 10 mM pyrophosphate buffer, 1.0 M NaCl, pH 7.4. Absorbance was monitored at 280 nm. Two solvent artefacts that were present in all traces, including blank runs, have been omitted for clarity.

stoichiometry of modification and yield data on the specificity of the reaction.

The reaction of 3,4-DCI with phosphorylase *b* was estimated by monitoring the changes in the absorbance of the reaction mixture at 325 nm which correlates with isocoumarin ring opening. Loss of absorbance at 325 nm due to spontaneous hydrolysis was subtracted from change in the absorbance at 325 nm in the presence of phosphorylase *b* to estimate the stoichiometry of 3,4-DCI modification. At 100 μM 3,4-DCI, inactivation proceeds over a 2 h period, and over the same period, one mol of phosphorylase *b* monomer interacts with approx. 5–6 mol equivalent of 3,4-DCI. Multiple sites of modification are consistent with the kinetics of inactivation (Fig. 2). Examination of the early stages of the reaction indicated that the second-order rate constant for reaction between phosphorylase *b* and 3,4-DCI was approx. 3.4 $M^{-1} \cdot s^{-1}$; substantially lower than for the serine proteases (human leucocyte elastase: 9820, bovine trypsin: 198, human leucocyte cathepsin G: 28 $mM^{-1} \cdot s^{-1}$ [2]). There are several weak nucleophilic sites on phosphorylase *b* that react with 3,4-DCI and which lead to progressive loss of activity.

The multiple modification of phosphorylase *b* by 3,4-DCI should introduce a number of anionic sites as a consequence of acylation by the open-ring form of the inhibitor. To test this, the phosphorylase *b* derivatives were resolved by Mono-Q FPLC (Fig. 4). Unmodified phosphorylase *b* did not bind Mono-Q under these buffer conditions and was eluted in 2 min. After incubation of phosphorylase *b* with 3,4-DCI, the phosphorylase *b* peak was reduced and new peaks were eluted at higher salt concentrations, consistent with an increasingly anionic character for phosphorylase *b*. This implies multiple sites of covalent modification of phosphorylase *b*, consistent with the stoichiometry of modification (Fig. 3).

The inactivation of glycogen phosphorylase *b* in vitro, involving multiple sites of weak reactivity, is more complex than that between 3,4-DCI and serine proteases. Since phosphorylase *b* possesses no special features that one might expect to promote interaction with a mechanism-based serine protease inhibitor, 3,4-DCI may also be capable of interacting with many other proteins and perhaps even other macromolecules. This would be particularly apparent if a high concentration of 3,4-DCI was used to elicit a rapid inactivation, to compensate for the instability of the inhibitor in some buffer systems, or to inactivate weakly-reactive proteases. Although 3,4-DCI is an effective serine protease inhibitor, we caution that examination of the effect of this inhibitor on other macromolecular components of an experimental system would be prudent.

Acknowledgement: N.M.R. is in receipt of an SERC Studentship.

REFERENCES

[1] Price, N.C. and Johnson, C.M. (1989) in: Proteolytic Enzymes – A Practical Approach (Beynon, R.J. and Bond, J.S. eds) pp. 163–180, Oxford University Press, Oxford.
[2] Harper, J.W., Hemmi, K. and Powers, J.C. (1985) Biochemistry 24, 1831–1841.
[3] Powers, J.C., Kam, C.-M., Narasimhan, L., Oletsysz, J., Hernandez, M.A. and Ueda, T. (1989) J. Cell. Biochem. 39, 33–46.
[4] Carney, I.T., Beynon, R.J., Kay, J. and Birkett, N. (1978) Anal. Biochem. 85, 321–324.
[5] Parvin, R. and Smith, R.A. (1969) Anal. Biochem. 27, 65–72.
[6] Beynon, R.J. (1985) Comp. Appl. Biosci. 1, 111–115.