RAPID PROTEOLYTIC REMOVAL OF PHOSPHOPEPTIDES AND PHOSPHORYLATABLE SITES FROM PROTEINS IN RAT LIVER CELL SAP

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1. Introduction

The activity of several enzymes is known to be regulated by phosphorylation–dephosphorylation reactions [1]. For some of these enzymes it has been shown that the phosphorylated sites are easily removed by proteolytic attack without inactivation of the enzymes [2–12]. The aim of the present investigation was to determine whether a removal of phosphorylated sites could be a common phenomenon for rat liver proteins that are substrates of protein kinases. It was considered that this study might be relevant for in vivo conditions, as it has been shown that the proteolytic sensitivity of proteins in vitro resembles that in vivo [13].

When cell sap is incubated with [^{32}P]ATP a considerable amount of [^{32}P]phosphate becomes incorporated into proteins due to phosphorylation by endogenous protein kinases [14]. Such a ^{32}P-labelled cell sap was incubated with subtilisin (EC 3.4.4.16), trypsin (EC 3.4.4.4) or α-chymotrypsin (EC 3.4.4.5). It was found that [^{32}P]phosphopeptides were released without proportionate degradation of the total protein, as estimated by determination of the remaining trichloroacetic acid precipitated protein.

2. Experimental

2.1. *Preparation of rat liver cell sap*

Rats were killed by cervical fracture and the liver was cut and washed twice in ice-cold 0.25 M sucrose containing 0.1 mM EDTA. All subsequent steps were performed at 4°C. The material was homogenized in 4 vol. same solution, using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 48 000 × *g* for 60 min. The supernatant was filtered through glass-wool and centrifuged at 100 000 × *g* for 60 min. After filtration through glass-wool the supernatant (50 ml) was chromatographed on a Sephadex G-50 column (4 × 24 cm) equilibrated and eluted with 20 mM triethanolamine–acetic acid buffer (pH 7.4). The protein eluted with the void volume was used in the phosphorylation and degradation experiments.

2.2. *Phosphorylation of cell sap*

Cell sap, 8 ml, were phosphorylated at 30°C with endogenous protein kinase and 0.1 mM [^{32}P]ATP (spec. act. 10^5 cpm/nmol) in the presence of 10 mM magnesium acetate and 25 μM cyclic 3′,5′-cyclic AMP (cAMP) unless otherwise stated. The [^{32}P]ATP was prepared by the method in [15] as modified [16]. After 2 min the incubation mixture was rapidly cooled to 4°C and chromatographed on a Sephadex G-50 column (2 × 16 cm) equilibrated and eluted with 20 mM potassium phosphate buffer (pH 7.5), containing 25 mM NaF and 0.1 mM EDTA. The phosphate and the fluoride inhibit the activity of phosphoprotein phosphatases [17]. The radioactive proteins were pooled.

2.3. *Treatment of ^{32}P-labelled cell sap with proteolytic enzymes*

Subtilisin (subtilopeptidase A: subtilisin Carlsberg; Sigma protease type P 5380) 0.2 ml in 10 mM potassiu

phosphate buffer (pH 7.0), trypsin (Trypsin-TPCK, Worthington) in 1 mM HCl or bovine α-chymotrypsin (Sigma) in 1 mM HCl was added to 2 ml phosphorylated cell sap at 30°C. At indicated times 0.04 ml samples were transferred to 2 ml 10% (w/v) trichloroacetic acid to interrupt the reaction and precipitate the protein. After centrifugation the radioactivity of the supernatants containing [^{32}P]phosphopeptides and [^{32}P]P_i was measured as Čerenkov radiation [18]. The pellets were dissolved in 0.2 ml 0.5 M NaOH and reprecipitated with 2 ml 10% trichloroacetic acid. After centrifugation the precipitates were dissolved in 0.5 ml 0.5 M NaOH and the radioactivity was measured. The protein content of the precipitates was determined as in [19], with bovine serum albumin as standard protein. The supernatants and pellets were analyzed for [^{32}P]SerP after acid hydrolysis in 2 M HCl for 20 h at 100°C and chromatographed on Dowex 50 [14,20]. All experiments described were repeated at least twice with similar results.

2.4. *Treatment of unlabelled cell sap with subtilisin followed by phosphorylation*

Cell sap, 1 ml, was incubated with subtilisin (10 μg/ml final concn) as described under section 2.3. At different times 0.2 ml samples were removed and 0.02 ml 10 mM phenylmethylsulfonyl fluoride was added to inactivate the subtilisin [12]. Control experiments in the absence of subtilisin were run in parallel. After 20 min at room temperature the samples were phosphorylated as described above. The reaction was interrupted by addition of 2 ml 10% trichloroacetic acid. The precipitates were washed 4 times as described above. The precipitates were analyzed for [^{32}P]SerP as described under section 2.3.

3. Results

3.1. *Proteolytic removal of [^{32}P]phosphopeptides from ^{32}P-labelled protein without a proportional degradation of total protein*

The radioactivity was easily removed from ^{32}P-labelled cell sap protein with the three proteolytic enzymes used (fig.1 A–C). By the analysis in [21] it was ascertained that the increase in trichloroacetic acid-soluble radioactivity did not represent [^{32}P]P_i. All increase in radioactivity of the supernatants between

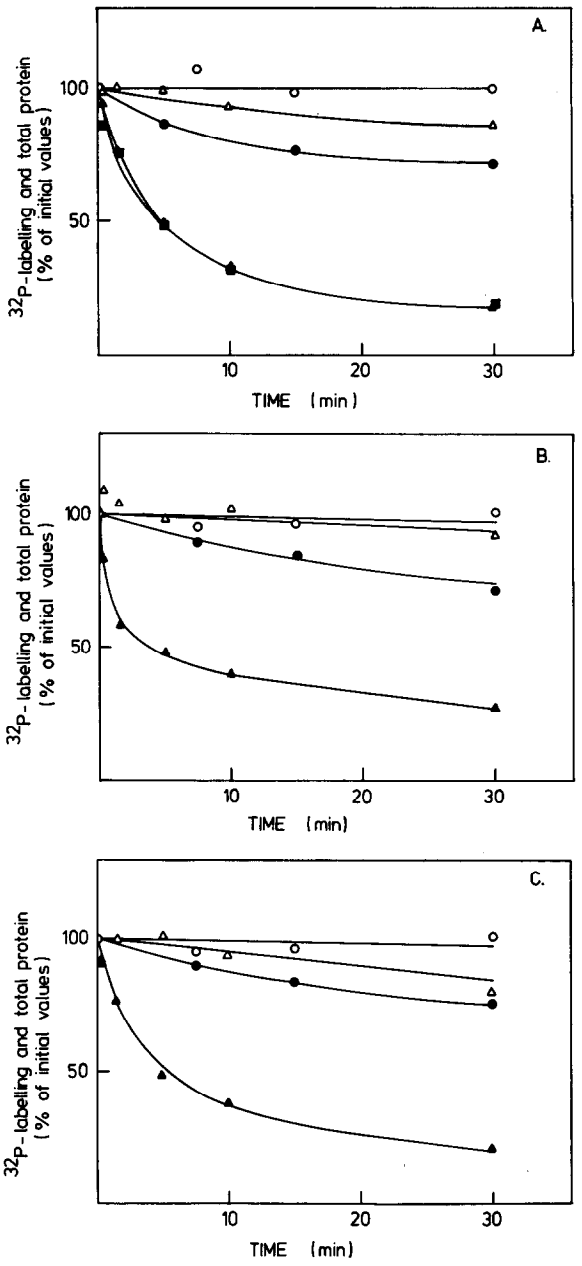

Fig.1. Digestion of ^{32}P-labelled rat liver cell sap protein with (A) 10 μg/ml subtilisin, (B) 20 μg/ml trypsin and (C) 100 μg/m α-chymotrypsin. The graphs show the remaining ^{32}P-labelling of the protein and the amount of protein after different digestion times. Open symbols represent the remaining protein as % initial value, and filled symbols the remaining radioactivity as % initial value. (○——○, ●——●) No proteolytic enzyme added. (△——△, ▲——▲) Proteolytic enzyme added. (■——■) The subtilisin digestion interrupted by 1 mM (final concn) phenylmethylsulfonyl fluoride.

zero time and the 30-min time point corresponded to [^{32}P]SerP. The amount of protein-bound [^{32}P] phosphate in the corresponding precipitates was estimated to be about 90%, after correction for losses and incomplete digestion during the acid hydrolysis. About 20% of the radioactive phosphate always remained bound to the protein. Under the conditions used the decrease in trichloroacetic acid-precipitable protein never exceeded 20%.

It was found that a lower concentration of subtilisin and a higher concentration of α-chymotrypsin were needed to achieve a similar rate of digestion to that of trypsin. Some proteolytic activity was also observed in the cell sap without addition of proteolytic enzyme.

3.2. *Release of [^{32}P]phosphopeptides as a function of the amount of protein-bound [^{32}P]phosphate*

It was of interest to see whether the sensitivity of proteins to proteolytic attack varied with the degree of ^{32}P-labelling, as the extent of phosphorylation may vary under different physiological conditions. To this end 2.4 ml portions of cell sap were incubated with 0.02, 0.1, 1.0 or 5.0 mM [^{32}P]ATP. The reactions were then chromatographed on 10 ml Sephadex G-50 columns. The chromatographies were completed in 5 min. The amount of [^{32}P]phosphate incorporation at the different concentrations of [^{32}P]ATP is given in table 1. There was no stimulation by cAMP at 1.0 mM and 5.0 mM [^{32}P]ATP.

Table 1
Incorporation of [^{32}P]phosphate into rat liver cell sap protein as a function of the concentration of [^{32}P]ATP, in the presence and absence of cAMP

[^{32}P]ATP (mM)	Pre-chromatography incubation (min)	[^{32}P]Phosphate incorporation (nmol/g liver)	
		cAMP absent	cAMP present
0.02	1	0.8	1.4
0.10	2	4.8	5.8
1.0	5	17	17
5.0	30	46	42

The cAMP concentration, when present, was 25 μM. The values are the means of two determinations. The specific activities of [^{32}P]ATP, in order of increasing concentrations, were 10^6, 10^5, 10^4 and 2000 cpm/nmol

Different radioactive protein samples (0.4 ml, about 3 mg) were digested for 2 min with subtilisin at a final concentration of 10 μg/ml or 20 μg/ml. Control experiments in the absence of proteolytic enzyme were run in parallel. Concerning the sensitivity of the [^{32}P]phosphorylated proteins to subtilisin, there was an apparently linear relationship between the [^{32}P]phosphopeptide release and the ^{32}P-labelling of the protein (data not given). This indicated that the various ^{32}P-labelled protein molecules were equally good as substrates for proteolytic enzymes. The release increased about 1.5 times when the higher concentration of subtilisin was used (data not given).

3.3. *Removal of [^{32}P]phosphopeptides from proteins phosphorylated by cAMP-dependent protein kinases*

As cAMP-stimulated protein kinases differ from other protein kinases with regard to substrate specificity [22], it was of interest to compare proteins phosphorylated in cAMP-dependent reactions with others phosphorylated independently of cAMP, regarding their sensitivity to subtilisin. Cell sap was labelled with 0.02 mM [^{32}P]ATP in the presence and absence of cAMP (table 1) and treated with subtilisin. The low [^{32}P]ATP concentration was chosen in order to obtain a high stimulation by cAMP. The results are shown in fig.2. The rate of release of [^{32}P]phosphopeptides was similar to the corresponding rates depicte in fig.1. This similarity also included the protein degradation curves (data not given). [^{32}P]Phosphopeptides were somewhat more easily released by subtilisin when the protein had been phosphorylated in the presence of cAMP.

3.4. *Removal of phosphate-accepting sites by limited proteolysis with subtilisin*

Phosphorylation can change the activity of enzymes [1] and affect the sensitivity of liver pyruvate kinase to proteolytic attack [23]. It therefore seemed of interest to see how sensitive are unphosphorylated phosphorylatable proteins to proteolytic enzymes in comparison with the phosphorylated proteins. Cell sap was digested with subtilisin and subsequently phosphorylated as described under section 2.

The material, preincubated in the absence of subtilisin, incorporated 5.5 nmol [^{32}P]P_i/g liver.

Proteins susceptible to phosphorylation were

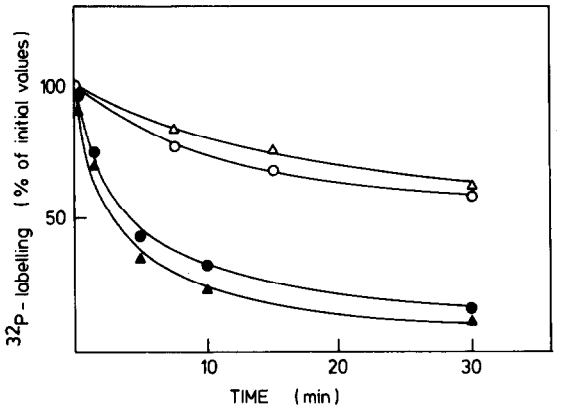

Fig.2. Subtilisin digestion of cell sap protein which had been labelled with [^{32}P]ATP in the presence and absence of cAMP. The concentration of cAMP when present during [^{32}P]-phosphorylation was 25 μM. The curves show the ^{32}P-labelling after different incubation times with or without subtilisin. Open symbols represent the remaining ^{32}P-labelling as % initial value when no proteolytic enzyme was added, and filled symbols the remaining ^{32}P-labelling as % initial value when subtilisin (10 μg/ml) was added. (○——○, ●——●) Cell sap phosphorylated in the absence of cAMP. (△——△, ▲——▲) Cell sap phosphorylated in the presence of cAMP.

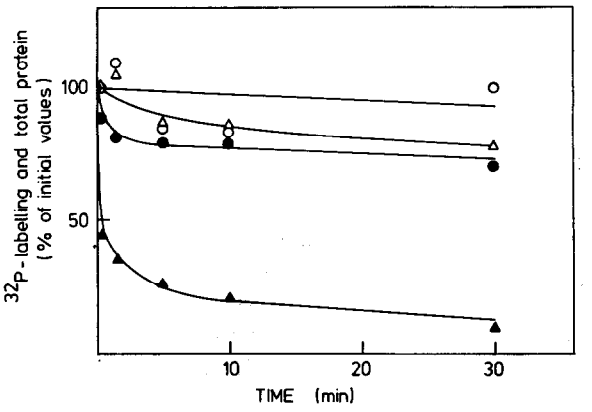

Fig.3. [^{32}P]Phosphorylation of rat liver cell sap protein after preincubation with subtilisin. The graph shows ^{32}P-labelling after different durations of preincubation. Open symbols represent the remaining protein as % initial value, and filled symbols the remaining radioactivity as % of the initial value. (○——○, ●——●) No subtilisin added. (△——△, ▲——▲) Subtilisin (10 μg/ml) added.

rapidly modified by limited proteolysis so that they lost their phosphorylatable sites (fig.3). The initial rate of modification was about twice as high as the initial rate shown in fig.1A. This was not due to the different techniques used, as the release of [^{32}P]-phosphopeptides did not change when the digestion of ^{32}P-labelled material with subtilisin was interrupted with phenylmethylsulfonyl fluoride and after 20 min precipitated with trichloroacetic acid. The residual activity of the endogenous protein kinases was about 80% after treatment with subtilisin for 30 min, when assayed with histone and phosvitin (4 mg/ml final concn) as substrates [24]. This indicated that unphosphorylated phosphorylatable sites were more sensitive to proteolysis than phosphorylated sites.

4. Discussion

It was shown that phosphorylatable parts of proteins were easily removed by addition of proteolytic enzymes. The unphosphorylated sites were about twice as sensitive to proteolytic attack as the phosphorylated ones in contrast to pyruvate kinase, which is 10 fold more sensitive in vitro to proteolysis in the inhibited phosphorylated form than in the unphosphorylated form [23].

The lability of proteins to proteolytic enzymes in vitro is correlated to a high turnover rate in vivo [13 As many regulatory enzymes have high turnover rates the observed rapid release of [^{32}P]phosphopeptides an phosphorylatable sites therefore supports the view that phosphoproteins in rat liver cell sap are regulatory enzymes or other regulatory proteins. This concept is also strengthened by the fact that some of the regulatory enzymes have been shown to be regulated by phosphorylation–dephosphorylation reactions [1]. The finding reported here that ^{32}P-labelled sites are sensitive to selective proteolysis also suggests that phosphorylation–dephosphorylation of proteins could be a mechanism for the regulation of protein degradation [13].

A proteolysis similar to that described in this paper could be an initial, rate-limiting step in vivo before the occurrence of further degradation. Such a type of mechanism has been suggested for the degradation of pyridoxal phosphate-containing enzymes [25].

In our experiments there was an endogenous

proteolytic activity acting on phosphorylated or phosphorylatable sites of proteins, suggesting that our in vitro findings have biological relevance.

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