# Hormonal effects on the phosphorylation of glycogen synthase in rat hepatocytes

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The effects of epinephrine and vasopressin on the phosphorylation state of glycogen synthase were studied using rat hepatocytes incubated with $^{32}P$. After the incubation with hormones, $^{32}P$-labeled glycogen synthase was isolated using antibodies against rat liver enzyme. The immunoprecipitate showed a single radioactive band ($M_{app}$ 88 kDa) when subjected to SDS-gel electrophoresis. Both epinephrine and vasopressin inactivated the enzyme and increased the $^{32}P$ content of glycogen synthase. Cleavage of the immunoprecipitate with CNBr yielded two major $^{32}P$-labeled fragments of $M_{app} \sim 27$ and 12 kDa. Both hormones increased the $^{32}P$ content of both fragments. These results prove that epinephrine and vasopressin increase the phosphate content of the enzyme promoting its phosphorylation at multiple sites.

*Glycogen synthase* *Hepatocyte* *Phosphorylation* *CNBr peptide* *Epinephrine* *Vasopressin* *Hormonal effect*

## 1. INTRODUCTION

Glycogen synthase is regulated through mechanisms of phosphorylation and dephosphorylation. In vitro glycogen synthase can be phosphorylated by different protein kinases at multiple sites. Phosphorylation causes the inactivation of the enzyme since it produces a change in the kinetic properties of glycogen synthase [1,2].

In vivo several hormones promote the inactivation of the enzyme. It is very important to establish the relationship between hormonal action and the phosphorylation state of the enzyme. Two different procedures have been used to approach this problem. The first is that followed in [3]. These authors purified glycogen synthase from rabbit skeletal muscle after injection of hormones to the animals and then they established a relationship between hormonal action and the phosphate content in different regions of the enzyme. In the second approach, tissues are incubated or perfused with [$^{32}P$]phosphate and the $^{32}P$ incorporated to glycogen synthase fragments after hormonal treatment is measured. The $^{32}P$ approach has been used in the study of the in vivo phosphorylation of glycogen synthase in rat heart [4,5] and in rat diaphragms [6].

However, no data were available for the liver enzyme. So far no direct evidence that the inactivation of the liver enzyme by glycogenolytic hormones is a consequence of the introduction of phosphates in glycogen synthase had been obtained.

This study was undertaken to establish a relationship between hormonal control of gycogen synthase activity and the phosphorylation state of the enzyme in rat hepatocytes. We prove that both epinephrine and vasopressin increase the $^{32}P$ content in two fragments of the enzyme indicating that

*Abbreviation:* $M_{app}$, apparent molecular mass

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the effect of these hormones is exerted through multisite phosphorylation of glycogen synthase.

## 2. MATERIALS AND METHODS

### 2.1. *Preparation of hepatocyte supernatants*

Suspensions of isolated parenchymal liver cells were prepared from 24 h starved male Wistar rats (180–220 g) as in [7]. Cells were finally resuspended in low phosphate (0.1 mM) Krebs bicarbonate buffer (pH 7.4), pre-gassed with $O_2(CO_2$ (19:1). Aliquots (5 ml, $3 \times 10^6$ cells/ml) were poured into stoppered vials, pre-incubated with $^{32}P_i$ (0.1–0.2 mCi/ml) for 60 min and then stimulated with either epinephrine or vasopressin. At the end of the hormonal incubations the content of each vial was centrifuged (3000 × g, 20 s) and the cell pellet immediately homogenized with 400 $\mu$l ice-cold medium containing 10 mM Tris (pH 7.0), 150 mM KF, 15 mM EDTA, 600 mM sucrose, 1 mM PMSF, 1 mM benzamidine, 50 $\mu$g/ml leupeptin and 50 mM $\beta$-mercaptoethanol. The presence of the proteinase inhibitors and a high concentration of sucrose was fundamental for avoiding proteolytic cleavage of the enzyme during homogenization.

Cell homogenates were centrifuged at 10 000 × g for 20 min and the obtained supernatants further centrifuged at 100 000 × g for 60 min to obtain cytosolic supernatants containing $^{32}$P-labeled glycogen synthase.

Glycogen synthase and phosphorylase activities were measured in supernatants of cells simultaneously subjected to the same procedure but without $^{32}$P being added to the incubation media.

### 2.2. *Immunoprecipitation of glycogen synthase*

Antibodies to rat liver glycogen synthase were raised in rabbits to which several injections of homogeneous rat liver glycogen synthase emulsified with Freund's complete adjuvant were given. Six weeks after the first injection the rabbits were bled and the antiserum was partially purified by precipitation with $(NH_4)_2SO_4$ (45% saturation).

Cytosolic supernatants were diluted 1:1 with 10 mM Triss (pH 7.0), 150 mM KF, 15 mM EDTA buffer. Then 175 $\mu$l of the partially purified antiserum were added to each aliquot. After 4 h of incubation at 4°C the mixture was centrifuged at 12 800 × g for 8 min and the pellets washed 3 times with 500 $\mu$l of the above-mentioned buffer. In this step, maintaining the preparation at low temperature was essential in avoiding proteolysis of the enzyme.

### 2.3. *Analytical procedures*

Cyanogen bromide treatment of $^{32}$P-labeled glycogen synthase immunopellets was carried out for 12 h at room temperature as in [8].

Samples for SDS–PAGE were lyophilized, resuspended in 0.5 ml water and lyophilized again. Finally, they were resuspended in 30 $\mu$l of sample buffer containing 40 mM Tris (pH 6.5), 5% SDS, 0.6 M $\beta$-mercaptoethanol, 20% sucrose and 0.0125% bromophenol blue as a marker. Electrophoresis was performed as in [9] and the gels were submitted to indirect autoradiography at −80°C. Developed films scanned at 546 nm.

Glycogen synthase activity was measured as in [10] and [11] and glycogen phosphorylase as in [12].

[$\gamma$-$^{32}$P]ATP specific radioactivity was determined essentially as in [13].

## 3. RESULTS

### 3.1. *Immunoprecipitation of glycogen synthase from hepatocyte extracts*

$^{32}$P-labeled glycogen synthase was rapidly purified from extracts of hepatocytes that had been incubated with $^{32}$P by immunoprecipitation. The antibodies were effective in removing glycogen synthase activity from extracts from control or hormone-treated hepatocytes. At the concentration of antibodies used, glycogen synthase activity was completely removed in control, vasopressin and epinephrine extracts (fig.1). However, the antibodies did not remove any of the glycogen phosphorylase activity present in the extracts.

### 3.2. *Electrophoretic analysis of $^{32}$P-labeled glycogen synthase from rat hepatocytes*

$^{32}$P-labeled glycogen synthase from extracts of control and hormone-treated hepatocytes was precipitated by the specific antibodies. Samples of the immunoprecipitates were resuspended after extensive washing and subjected to SDS-electrophoresis. Regardless of the hormone treatment, all of the radioactivity was present in a single band which had the same mobility as purified rat liver

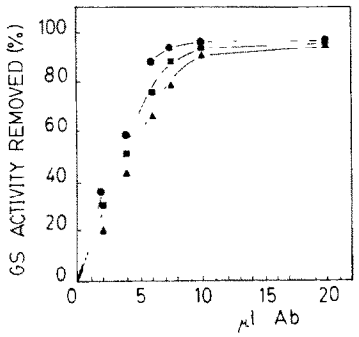


Fig.1. Binding of antibodies to glycogen synthase from extracts of control and hormonally-treated hepatocytes. Hepatocytes were incubated with saline (●), $10^{-5}$ M epinephrine (▲) or $10^{-7}$ M vasopressin (■). Extracts were prepared and aliquots were added with increasing concentrations of control serum or anti-glycogen synthase antiserum and incubated at 4°C for 4 h. Then samples were centrifuged and glycogen synthase activities (in the presence of 6.6 mM glucose-6P) were measured in the supernatants. The results presented represent percentages of glycogen synthase activities removed by the antibodies.

glycogen synthase labeled with $^{32}P$ by rat incubation with [$\gamma$-$^{32}P$]ATP and the catalytic subunit of the rat liver cAMP-dependent protein kinase. The radioactive band showed an $M_{app}$ of 88 kDa. If proper care was not taken in the preparation of the extracts or in the immunoprecipitation procedure, a second $^{32}P$-band with an $M_{app}$ of 80 kDa could be observed, which corresponded to a proteolytic fragment of the main subunit (fig.2). The presence of sucrose in the homogenization media and temperature control were the most important factors in this regard.

### 3.3. *CNBr cleavage of the $^{32}P$-labeled glycogen synthase*

Treatment of the $^{32}P$-immunoprecipitates with CNBr resulted in the formation of two $^{32}P$-fragments. The band of lower mobility (peak II) had an $M_{app}$ of 27 kDa and the faster moving band (peak I) had an $M_{app}$ of 12 kDa. These fragments corresponded with those obtained after CNBr cleavage of the purified rat liver glycogen synthase phosphorylated with either the catalytic subunit of the cAMP-dependent protein kinase or the casein kinase-1 purified from rat liver.

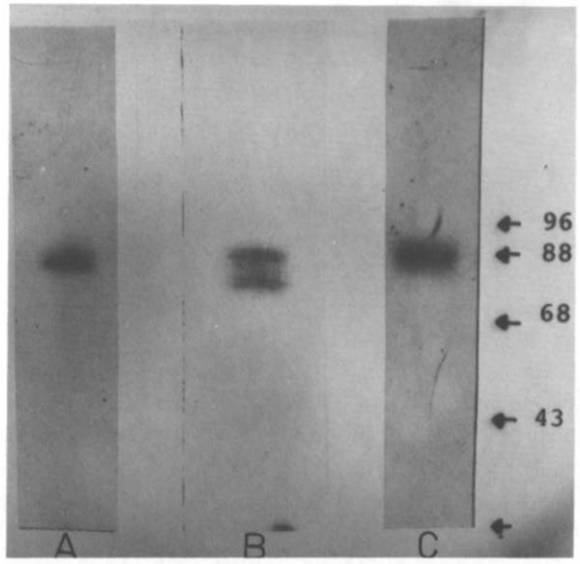


Fig.2. Electrophoretic analysis of $^{32}P$-labeled glycogen synthase. Immunoprecipitates of glycogen synthase were submitted to electrophoresis in 7% acrylamide gels and then autoradiograms were obtained. (A,B) Immunoprecipitates from extracts of $^{32}P$-labeled hepatocytes prepared in presence (A) or absence (B) of 600 mM sucrose. (C) Immunoprecipitate of purified rat liver glycogen synthase phosphorylated in vitro by the catalytic subunit of rat liver cAMP-dependent protein kinase. Molecular masses of standard proteins are shown on the right.

### 3.4. *$^{32}P$-labeling of the ATP by incubation of rat hepatocytes with [$^{32}P$]phosphate*

Hepatocytes were incubated with [$^{32}P$]phosphate for 1 h. The [$\gamma$-$^{32}P$]ATP specific activity reached a plateau after approx. 45 min of incubation (not shown) and a specific radioactivity of 25–30 cpm/pmol was achieved. No change in the specific radioactivity of [$^{32}P$]ATP could be detected by subsequent hormonal treatment.

### 3.5. *Hormonal effects on the glycogen synthase activity of rat hepatocytes*

Both vasopressin ($10^{-7}$ M) and epinephrine ($10^{-5}$ M) caused the inactivation of glycogen synthase. This effect was magnified when measured by the low glucose 6-P/high glucose 6-P activity ratio method (table 1). The time of incubation with the hormones was fixed at 8 min for vasopressin and 5 min for epinephrine, as in previous experiments it was observed that effects

Table 1

Effects of epinephrine and vasopressin on glycogen synthase

| | GS activity ratio (0.25 mM G6P/10 mM G6P | CNBr $^{32}$P-fragments | | Peak II/Peak I |
|---|---|---|---|---|
| | | Peak I | Peak II | |
| Control | 44 ± 0.9 | 100 | 100 | 2.2 ± 0.3 |
| Vasopressin | 35 ± 1.3 | 390 ± 41 | 296 ± 51 | 1.4 ± 0.1 |
| Epinephrine | 36 ± 1.5 | 403 ± 29 | 290 ± 35 | 1.5 ± 0.2 |

Hepatocytes were incubated with or without hormones in duplicate samples with or without $^{32}$P before extracts were prepared. Glycogen synthase low G6P/high G6P activity ratio was measured in extracts of cells not incubated with $^{32}$P. In extracts of $^{32}$P-labeled cells glycogen synthase was immunoprecipitated, treated with CNBr and subjected to electrophoresis. Autoradiograms of gels were scanned and the peaks corresponding to the two CNBr fragments were integrated. Results are expressed as a percentage of the values of the peaks from control cells. Results are means ± SE of 5 experiments

of these hormones were maximal at the above indicated times. Similarly, the concentration used corresponded to maximal doses.

### 3.6. *Hormonal effects on the $^{32}$P content of both CNBr fragments of glycogen synthase*

$^{32}$P-labeled glycogen synthase was immunoprecipitated from extracts of control and hormone-treated hepatocytes and subjected to SDS-electrophoresis. Autoradiograms were prepared and scanned as indicated. Incubation of the cell with either vasopressin or epinephrine resulted in an increase of the peak of $^{32}$P-labeled glycogen synthase. In order to determine if these hormones preferentially affected the phosphorylation of different CNBr fragments of the enzyme, autoradiograms from gels of samples that had been treated with CNBr were scanned.

Incubation of the hepatocytes with both epinephrine or vasopressin resulted in an increase in the total amount of the radioactivity present in both CNBr fragments (fig.3). Radioactivity in peak II was increased about 3 times and that in peak I about 4 times. Therefore the ratio of the radioactivity associated with peak II to that in peak I, which was approx. 2.2:1 in the control, decreased to about 1.4:1 in hormone-treated hepatocytes (table 1).

## 4. DISCUSSION

The results presented prove that rat hepatocyte glycogen synthase is phosphorylated in vivo at

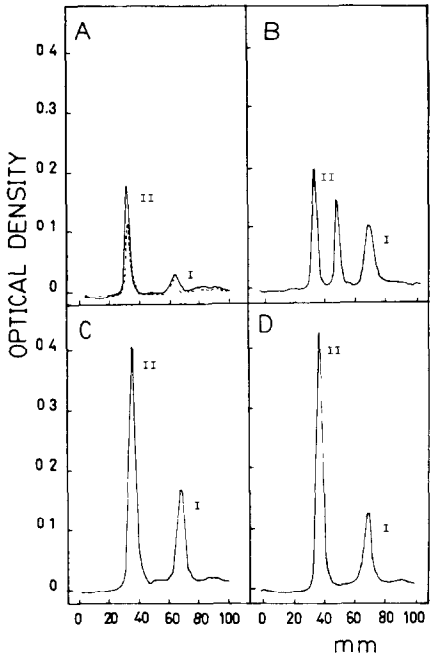


Fig.3. Electrophoretic analysis of the CNBr fragments of $^{32}$P-labeled glycogen synthase. Samples of the immunoprecipitates were cleaved with CNBr before being subjected to electrophoresis in 15% acrylamide gels. Autoradiograms were obtained and then scanned. (A,C,D) Immunoprecipitates of $^{32}$P-labeled glycogen synthase obtained from control (A), epinephrine (C) and vasopressin (D) treated hepatocytes. (B) The pattern of CNBr cleavage products of partially proteolyzed glycogen synthase. The dotted lines in (A) correspond to CNBr fragments of purified glycogen synthase phosphorylated in vitro with the catalytic subunit of cAMP-dependent protein kinase.

several sites and that its state of phosphorylation is increased by hormones which inactivate the enzyme.

Our experimental approach was to incubate hepatocytes with $^{32}P$ followed by immunoprecipitation of the enzyme. This approach which has been useful in the study of glycogen synthase from rat heart [4,5] or rat diaphragm [6] has also been very useful for the liver system. In this case we have used antibodies raised in goat against the rabbit muscle enzyme. The $^{32}P$-labeled glycogen synthase immunoprecipitated from rat hepatocytes had an $M_{app}$ of 88 kDa. This value was identical to that of homogeneous rat liver glycogen synthase phosphorylated in vitro by different protein kinases. This immunoprecipitation was highly specific since the $M_{app}$ 88 kDa was the only radioactive band obtained and the antibody did not eliminate any of the phosphorylase activity present in the extracts. However, maximal care had to be taken to avoid proteolysis of the enzyme. If not, a proteolytic band of $M_{app}$ 80 kDa could easily appear.

Treatment of glycogen synthase with CNBr gives rise to two $^{32}P$-labeled fragments. These fragments coincide with those obtained by CNBr cleavage of the purified rat liver glycogen synthase phosphorylated in vitro by different protein kinases. Therefore in vivo [$^{32}P$]phosphate is introduced in the same regions of the glycogen synthase molecule which are also phosphorylated in vitro. It is worth noting that the muscle enzyme is also phosphorylated in vitro as well as in vivo at two CNBr fragments.

An important conclusion of this paper is that both vasopressin and epinephrine increase the phosphate content in both CNBr fragments and although more phosphate was actually introduced in the largest fragment (II), on a percentage basis the increase in the radioactivity in the smaller CNBr fragment was more pronounced. Therefore these results suggest multiple phosphorylation of liver glycogen synthase after hormonal treatment. A similar conclusion was reached regarding muscle in [3] and [6].

Experiments in progress in our laboratory on the site specificity of several rat liver protein kinases acting on rat liver glycogen synthase will permit a comparison between the sites phosphorylated in vivo in response to hormonal treatment and those phosphorylated in vitro by the different protein kinases.

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