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The Importance of Arginyl Residues for Phosphorylation of Rat Liver Cell Sap Proteins

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Arginyl residues in phosvitin, histone and cell sap protein were blocked by 1,2-cyclohexanedione, resulting in markedly impaired phosphorylation of histone and cell sap. Interestingly, the phosphate incorporation into phosvitin was *not* changed by this treatment.

Intact arginyl residues in the protein kinase substrates seemed to be essential for more than half of the cell sap phosphorylation at 5 mM ATP.

Furthermore *both* phosvitin kinase and histone kinase activities in cell sap were inhibited by arginyl residue blockade, indicating that these enzymes had functional arginyl residues.

The increasing number of identified substrates for cyclic AMP-stimulated protein kinase has given rise to the question of what determines the specificity in these phosphorylation reactions. The primary structure at the phosphate-accepting site seems to be of great importance, with the presence of at least one arginyl [1–5] or lysyl [6] residue at a certain small distance from the amino acid residue to be phosphorylated. Evidence for an essential arginine recognition site on cyclic AMP-dependent rabbit skeletal muscle protein kinase has recently been published.[7] Concerning substrates for cyclic AMP-independent protein kinases, there are some indications that lysyl residues are needed for phosphorylation by phosvitin kinase.[8] Furthermore, for the phosphorylation of peptides by phosphorylase b kinase both an arginyl and a lysyl residue are important.[9]

Previous studies have to a great extent been dealing with the phosphorylation of small peptides derived from known substrates for protein kinases.[1–4,6,9] In order to investigate the importance of arginine for phosphorylation from a more general point of view, phosphorylation experiments were performed with rat liver cell sap treated with the specific arginine blocking reagent 1,2-cyclohexanedione.[10] For comparison the effects of 1,2-cyclohexanedione on histone and phosvitin were also examined.

EXPERIMENTAL

Materials

Bovine serum albumin, calf thymus histone (Sigma type II-A), phosvitin, dithiothreitol, cyclic AMP and ATP were purchased from Sigma and 1,2-cyclohexanedione from Aldrich. Sephadex G-50 fine was obtained from Pharmacia, Uppsala. (^{32}P)ATP was a product of New England Nuclear, Boston. Cyclic AMP-stimulated protein kinase from rat liver was prepared through the hydroxylapatite step as described by Titanji *et al.*[11]

Preparation of rat liver cell sap

Male Sprague-Dawley rats, fed on ordinary laboratory chow, were killed by cervical fracture and the liver (average weight 15 g) was cut into pieces and washed in ice-cold 0.25 M sucrose containing 1 mM EDTA and 0.1 mM dithiothreitol. All subsequent steps were performed at 4 °C. The liver pieces were homogenized in four volumes of the same solution, using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 43 000 *g* for 20 min, whereafter the supernatant was filtered through glass-wool and centrifuged at 100 000 *g* for 60 min. The supernatant emerging from this run was chromatographed on

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a Sephadex G-50 column equilibrated and eluted with 0.25 M sodium borate, pH 8. The protein eluted with the void volume was divided into smaller portions and stored frozen. One ml of this cell sap corresponded to 0.20 – 0.25 g of original tissue (wet weight).[12] The samples were not thawed and refrozen.

Treatment with 1,2-cyclohexanedione

Histone and phosvitin were dissolved in 0.25 M sodium borate, pH 8, to concentrations of 10 mg/ml and 25 mg/ml, respectively. Prior to use the cell sap was mixed with EDTA, EGTA, NaF and phenylmethylsulfonyl fluoride to final concentrations of 0.11 mM, 0.11 mM, 28 mM and 0.56 mM respectively, the combined volumes of these additives amounting to 9 % of the mixture.

1,2-Cyclohexanedione solution, 0.5 M in 0.25 M sodium borate, pH 8, was prepared and used within 1 – 2 h. To the ice-cold protein samples 0.1 volume of this solution (thus 50 mM 1,2-cyclohexanedione) was added, whereafter the tubes (containing 0.5 – 2 ml) were immediately transferred to a water-bath with a temperature of 37 °C. On addition of the reagent the histone solution became turbid, although there was no visible precipitation of phosvitin or cell sap.

Phosphorylation experiments

(^{32}P)Phosphate incorporation into histone, phosvitin and cell sap incubated with 1,2-cyclohexanedione. During incubation with 1,2-cyclohexanedione 50 µl samples were removed and pipetted into prewarmed (30 °C) tubes containing sodium borate buffer, pH 8, magnesium acetate, (^{32}P)ATP (80 000 – 100 000 cpm/nmol) and cyclic AMP to final concentrations of 0.23 M, 10 mM, 0.1 mM and 25 µM, respectively. In some experiments, 50 µl of cell sap, not treated with 1,2-cyclohexanedione but five times more diluted, were added to provide extra protein kinase. The phosphorylation was performed at a final volume of 0.5 ml, thus the concentration of 1,2-cyclohexanedione decreased to 5 mM. This concentration of the reagent did not affect the 5 min phosphate incorporation into cell sap. Phosphorylation was interrupted after 5 min by the addition of 2 ml of ice-cold 10 % (w/v) trichloroacetic acid, and 1 mg of bovine serum albumin was added. After centrifugation, the supernatant was discarded and the protein was dissolved in 0.5 ml 0.2 M NaOH and reprecipitated with 10 % trichloroacetic acid containing 50 mM H_3PO_4. This was repeated once, after which the protein was dissolved in 1 ml NaOH. The radioactivity was then measured as Cerenkov radiation.[13] For comparison, samples were processed in the same way as described here but with no addition of 1,2-cyclohexanedione in the incubation at 37 °C. It was ascertained that the incorporation of phosphate was linear with time under the incubation conditions used.

Some experiments were also performed with purified cyclic AMP-stimulated rat liver protein kinase. This was added to the phosphorylation incubation mixtures in amounts capable of transferring 64 pmol of (^{32}P)phosphate per min from 0.1 mM (^{32}P)ATP into mixed histones (4 mg/ml) at pH 7.

Protein kinase activity in cell sap during incubation with 1,2-cyclohexanedione. Cell sap was incubated with 50 mM 1,2-cyclohexanedione as described above and the protein kinase activity (at 0.1 mM (^{32}P)ATP *etc.* as above) of 50 µl samples on 0.25 mg histone or 0.75 mg phosvitin, respectively, was estimated.

Stability test of 1,2-cyclohexanedione effect. 1,2-Cyclohexanedione-treated (90 min, 37 °C) histone, phosvitin and cell sap samples were dialyzed overnight at 4 °C against 3 changes of 0.25 M sodium borate, pH 8, containing 0.1 mM EDTA. 0.1 mM EGTA and 25 mM NaF. The samples were then phosphorylated and the phosphate incorporation (5 min, 0.1 mM (^{32}P)ATP) was compared with the incorporation into undialyzed samples.

Maximal (^{32}P)phosphate incorporation into cell sap after treatment with 1,2-cyclohexanedione. Phosphorylation experiments with the 1,2-cyclohexanedione-treated, dialyzed cell sap were also performed at 5 mM (^{32}P)ATP (9000 cpm/nmol) in 0.20 M sodium borate, pH 8, 0.08 mM EDTA, 0.08 mM EGTA and 20 mM NaF. As before, the amount of cell sap added to provide protein kinase corresponded to 1/5 of the 1,2-cyclohexanedione-treated cell sap. Samples of 100 µl were removed from the incubation mixture at different times and precipitated with 10 % trichloroacetic acid. The precipitates were washed and the phosphate incorporation determined as described above.

All experiments were repeated at least twice, with similar results.

Amino acid analysis

1,2-Cyclohexanedione-treated and untreated samples were dialyzed as described above and thereafter against 70 % HCOOH, and were then dried and submitted to amino acid analysis. The material was hydrolyzed for 24 h at 110 °C in sealed ampoules containing 6 M HCl and 1 % phenol. A one-column Durrum amino acd analyzer (Durrum D-500) was used.

RESULTS AND DISCUSSION

1,2-Cyclohexanedione effect on (^{32}P)phosphate incorporation. As an introduction to the experiments with cell sap, which contains a mixture of different protein kinases and their

substrates, the effect of the arginine blocking reagent on the phosphorylation of purified proteins was studied. Histone and phosvitin were used, representing examples of substrates for cyclic AMP-dependent and cyclic AMP-independent protein kinases, respectively. It appeared that during a 30 – 40 min period, histone incubated with 1,2-cyclohexanedione gradually incorporated less phosphate (the incorporation being reduced to about 20 % of the initial value), while the phosphorylation of phosvitin was not affected (Fig. 1).

The stability of the cell sap protein kinase activity in 50 mM 1,2-cyclohexanedione was examined, using histone and phosvitin as substrates. These kinase activities were markedly reduced (Fig. 2). If the reagent was excluded from the incubation medium, 90 % of the initial protein kinase activities remained after 70 min (not shown).

In cell sap samples incubated with 1,2-cyclohexanedione, the (^{32}P)phosphate incorporation was reduced to about 15 % (Fig. 3), probably resulting from effects on endogenous protein kinases (*cf.* Fig. 2) as well as on phosphorylatable proteins. In order to get more

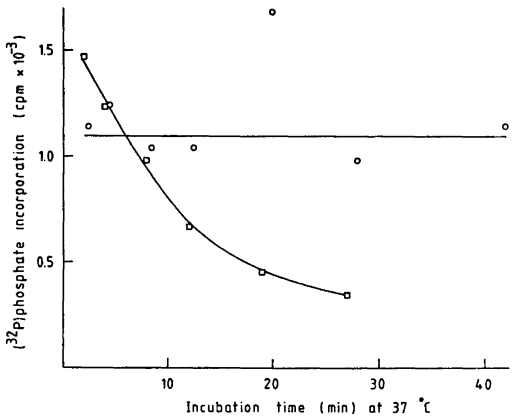

Fig. 1. Phosphorylation of phosvitin and histone, incubated with 1,2-cyclohexanedione. During incubation with 1,2-cyclohexanedione at 37 °C, samples were removed and phosphorylated for 5 min at 30 °C with 0.1 mM (^{32}P)ATP (78 000 cpm/nmol) and "fresh" cell sap (=not treated with 1,2-cyclohexanedione). (^{32}P)Phosphate incorporation (corrected for endogenous phosphorylation of added cell sap) into phosvitin (○) and histone (□).

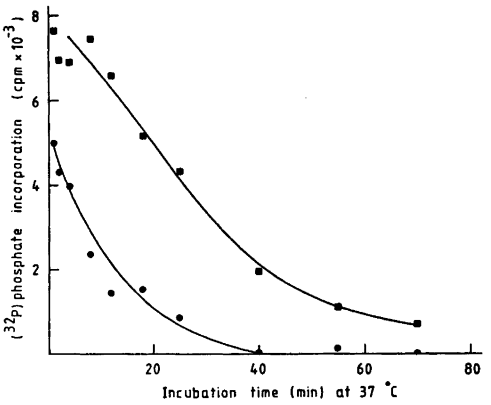

Fig. 2. Inactivation of phosvitin kinase and histone kinase in cell sap by 1,2-cyclohexanedione. During incubation of cell sap with cyclohexanedione at 37 °C samples were removed and phosphorylated for 5 min at 30 °C and 0.1 mM (^{32}P)ATP (88 000 cpm/nmol) with the addition of „fresh" phosvitin or histone. (^{32}P)Phosphate incorporation (corrected for endogenous phosphorylation in cell sap) into phosvitin (●) and histone (■).

clear-out evidence of a 1,2-cyclohexanedione effect on protein kinase substrates in cell sap, the experiments of Fig. 3 were repeated with purified cyclic AMP-stimulated rat liver protein kinase. In contrast to unfractionated cell sap there was very little endogenous (^{32}P)phosphate incorporation into this protein kinase preparation. The results (not shown) were similar to those presented in Fig. 3. In both cases the difference between the endogenous cell sap phosphorylation and the phosphorylation in the presence of added protein kinase gradually diminished, suggesting a decreasing amount of rapidly phosphorylated sites. As a control, cell sap was also incubated at 37 °C in the absence of the reagent (Fig. 3). This material was phosphorylated to a diminishing extent too, the incorporation being reduced to 60 – 70 % of the initial value. Thus, most of the reduction in phosphate incorporation into cell sap incubated with 1,2-cyclohexanedione seemed to be due to the reagent and not to other factors. Furthermore, the diminished phosphate incorporation into cell sap incubated in the absence of 1,2-cyclohexanedione might represent more than the correct blank to the experiment performed in the presence of the

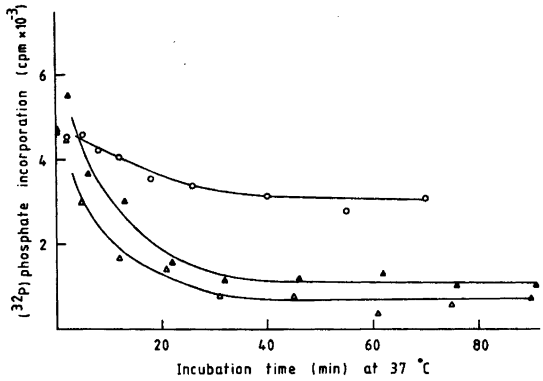

Fig. 3. Phosphorylation of cell sap, incubated with 1,2-cyclohexanedione. During incubation of cell sap in the presence (△,▲) or absence (○) of 1,2-cyclohexanedione at 37 °C, samples were removed and phosphorylated for 5 min at 30 °C and 0.1 mM (^{32}P)ATP (99 000 cpm/nmol) with (filled symbols) or without (open symbols) addition of "fresh" cell sap.

reagent. Prephosphorylated cell sap samples lost 8, 15 and 22 % of their trichloroacetic acid insoluble (^{32}P)radioactivity when incubated for 60 min at 37 °C in the presence of 50 mM and 10 mM 1,2-cyclohexanedione and in the absence of this reagent, respectively. 1,2-Cyclohexanedione obviously protected phosphorylated sites, for example by inactivation of phosphoprotein phosphatases or proteolytic enzymes.

In order to examine whether the impairment of phosphorylation could be overcome by longer incubation times and a higher ATP concentration, cell sap was treated with 1,2-cyclohexanedione (to inactivate protein kinases and affect protein kinase substrates), dialyzed (to avoid inactivation of added protein kinase by the reagent during a prolonged incubation time) and used for the experiments illustrated in Fig. 4. The maximal phosphate incorporation into cell sap (corresponding to 30 nmol of (^{32}P) phosphate per g liver wet weight) at 5 mM ATP was reduced to about 20 % by this treatment. Fresh cell sap, when added, increased the phosphate incorporation into the 1,2-cyclohexanedione-treated cell sap to 30 – 40 % of the control. At this value a plateau was reached, indicating that no more phosphorylation was possible.

Modification of arginyl residues. The impairment of phosphorylation by treatment with 1,2-cyclohexanedione could not be reversed

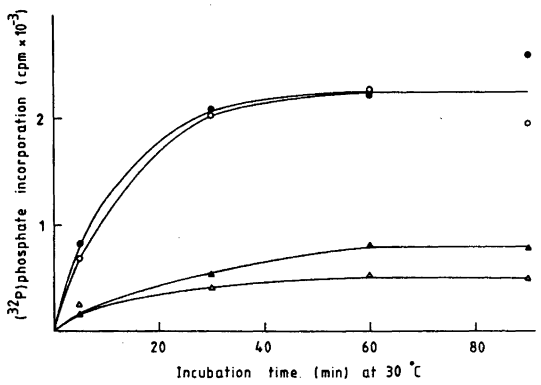

Fig. 4. (^{32}P)phosphate incorporation at 30 °C and 5 mM (^{32}P)ATP (9 000 cpm/nmol) into 1,2-cyclohexanedione-treated (△,▲) and untreated (○,●) cell sap. The values are corrected for endogenous (^{32}P)phosphate incorporation into "fresh" cell sap, where this was added (filled symbols).

by dialysis, indicating that the reagent had caused a stable change of the protein.

In the amino acid analysis the value for (only) arginine differed between the 1,2-cyclohexanedione-treated sample and the control.[10] As compared to the control value (100 %) there was an arginine reduction to 50 %, 46 % and 64 % for cell sap, histone and phosvitin, respectively. Since the main intention was to see whether arginine diminished in amount, indicating that the expected reaction with 1,2-cyclohexanedione had taken place, only one analysis of each sample was performed and no additions with the purpose of avoiding spontaneous regeneration of arginine during hydrolysis (18–20 % in Ref. 10) were made.

Conclusions. A substantial amount of arginyl residues in phosvitin, histone and cell sap protein could be blocked by cyclohexanedione. This resulted in a considerable inhibition of the phosphorylation of histone and cell sap proteins, while the phosphate incorporation into phosvitin was not changed.

Thus, histone seemed to belong to those protein kinase substrates that contain arginyl residues essential for phosphorylation. Phosvitin, one example of substrates for cyclic AMP-independent protein kinases, was phosphorylated to the same extent whether or not arginyl residues were blocked. The phosphorylation of rat liver cell sap proteins by endogenous protein kinases and by purified cyclic AMP-stimulated protein kinases at 0.1 mM ATP decreased on blocking of arginyl residues in cell sap.

This might reflect different criteria for rendering proteins substrates for phosvitin kinases on the one hand and cyclic AMP-stimulated protein kinases on the other.

The maximal phosphorylation of arginine-blocked cell sap proteins by (active) cell sap protein kinases at 5 mM ATP reached a considerably lower plateau than the control, indicating that many previously phosphorylatable sites had become inaccessible. Under these conditions intact arginyl residues seemed to be essential for more than half of the cell sap protein phosphorylation.

These findings of the need for intact arginyl residues confirming phosphorylation experiments with small peptides support the idea that peptides can serve as good model substrates when elucidating structural requirements for phosphorylation.

However, some cell sap protein kinases might have functional arginyl residues since both phosvitin kinase and histone kinase activity was inhibited by blocking of arginyl residues. This indicates that arginyl residues play a greater role than previously described in interactions between the protein kinase and its substrate.

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