Upsala J Med Sci 92: 217–232, 1987

Studies on the Regulation of Rat Liver Pyruvate Kinase and Fructose-1,6-Bisphosphatase

Minireview based on a doctoral thesis

Kristina Nilsson Ekdahl

Department of Medical and Physiological Chemistry, Biomedical Center, Uppsala University, Uppsala, Sweden

INTRODUCTION

In the living cell there is a constant need for rapid adjustment of various anabolic and catabolic reactions in response to extracellular stimuli such as hormones.

Control of metabolic pathways can occur at several different levels using mechanisms such as substrate and cofactor availability, product removal, feedback regulation, allosteric effects, covalent modifications of the enzyme such as phosphorylation or proteolytic modification, and induction or repression of the total amount of the enzyme (56).

REGULATORY PHOSPHORYLATION

Hormones transmit information to the interior of the cell by activating transmembrane signaling systems. About half of the hormones found in mammals, e. g. glucagon and epinephrine, act by increasing the concentration of 3',5' cyclic-AMP (cAMP) (27, 70).

cAMP regulates protein kinase activity thus altering the phosphorylation states and biological properties of many intracellular enzymes (for a review of the current knowledge see 10).

The first enzyme of which the activity was reported to be regulated by phosphorylation was glycogen phosphorylase (73). The rapid increase in the number of enzymes reported to serve as substrates in phosphorylation-dephosphorylation reactions has made it necessary to formulate certain criteria for establishing that the phosphorylation-dephosphorylation reactions have biological significance (44, 56), here with special reference to cAMP-dependent protein kinase:

1. The existence of a substrate for cAMP-dependent protein kinase which participates in a metabolic pathway influenced by cAMP. The rate of phosphorylation in vitro must be compatible with the speed with which the process occurs in vivo.
2. A reversible change in enzyme activity in vitro caused by phosphorylation-dephosphorylation catalyzed by cAMP-dependent protein kinase and phosphoprotein

phosphatase respectively.

3. A reversible change in enzyme activity in vivo in response to cAMP.
4. The amino acid residue(s) phosphorylated in response to a hormone in vivo must be the same as the residue(s) phosphorylated in vitro by cAMP-dependent protein kinase.

CYCLIC AMP-DEPENDENT PROTEIN KINASE

Protein kinases, first described in 1954 (6), catalyze the transfer of phosphate groups from nucleoside triphosphate, ATP or GTP, to a protein substrate. The phosphorylatable amino acid residues in the substrate molecule are serine, threonine and tyrosine. The activity of several protein kinases is dependent on specific agents such as cyclic nucleotides, divalent cations and phospholipids (13, 45, 74). Here only cAMP-dependent protein kinase (cAMP-PK) will be discussed.

cAMP-PK was first described in 1968 (46, 81) and consists of two groups, types I and II. Both types are composed of two regulatory and two catalytic subunits. The enzyme is activated when cAMP binds to the regulatory subunits thus releasing the active catalytic subunits (45).

cAMP-PK phosphorylates serine and threonine residues with ATP as phosphate donor and Mg^{2+} as cofactor. Synthetic peptides have been used as substrates for cAMP-PK and such studies have revealed that the presence of basic amino acid residues on the N-terminal side from the phosphorylation site is essential (42, 82, 83).

cAMP-PK has a broad specificity for protein substrates (25) and has been reported to phosphorylate e.g. ATP citrate-lyase (35), fibrinogen (24), fructose-1,6-bisphosphatase (64), fructose-2,6-bis-phosphatase / 6-phosphofructo-2-kinase (21, 62), glycogen synthase (66), histones H1, H2A and H2B (33, 71), hormone sensitive lipase (2, 41), Na,K-ATPase (53), phosphofructokinase (5), phosphorylase b kinase (72) and pyruvate kinase, types L and R (47, 49).

PYRUVATE KINASE

Pyruvate kinase (E.C. 2. 7. 1. 40.) catalyzes the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP, respectively, this being the last reaction in the glycolysis chain of reactions.

The activity of the L-type pyruvate kinase in liver is regulated by phosphorylation and dephosphorylation in vivo (for reviews see 24, 26) in responce to hormones such as glucagon and epinephrine (54, 77).

In vivo phosphorylation of pyruvate kinase will decrease its activity and at least partially prevent futile cycling between phosphoenolpyruvate and pyruvate by supressing glycolysis and favoring gluconeogenesis.

Hepatic L-type pyruvate kinase: Separation of unphosphorylated, phosphorylated and proteolytically modified in vivo forms.

Since the activity of rat liver pyruvate kinase of the L-type is regulated by reversible phosphorylation *in vivo* the proportions between phosphorylated and unphosphorylated pyruvate kinase could be expected to differ in the liver of animals in different nutritional states.

The phosphorylated and unphosphorylated forms of an enzyme generally differ very little in isoelectric point, pI, (11) and it is hard to separate the two forms by ion-exchange chromatography. In order to overcome these difficulties the chromatofocusing system was used (14). Cell sap from livers of rats which had been fed *ad libitum*, starved for 48 h, or which had been fed on a diet containing 40 % fructose, was subjected to chromatography on DEAE-cellulose. This procedure removed about 95 % of the cellular proteins. The remaining proteins, including pyruvate kinase of the L-type, were separated by chromatofocusing, a procedure which yielded three fractions exhibiting pyruvate kinase activity and having apparent pI's of 5.0, 5.2 and 5.3, respectively.

These forms were identified as phosphorylated, unphosphorylated and proteolytically degraded pyruvate kinase, respectively, on the grounds of data on kinetics and phosphorylation. The identifications of the three forms of pyruvate kinase were further corroborated by separately subjecting unphosphorylated, phosphorylated and proteolytically digested pyruvate kinases to chromatofocusing under the same conditions. Since it was impossible to tell which protease had generated the third form of pyruvate kinase, an enzyme that had been degraded by a calcium activated protease was used as a model of digested pyruvate kinase. The calcium activated protease is known to remove a small peptide containing the phosphorylation site from the L-type of pyruvate kinase (12). The phosphorylated, unphosphorylated and digested pyruvate kinases eluted at pH 5.0, 5.2 and 5.3, respectively, and the degradation product showed an apparent K_m which was higher than that of both phosphorylated and unphosphorylated pyruvate kinase. It was therefore concluded that the third pyruvate kinase fraction found in rat liver cell sap represented a form which had been proteolytically modified, and also that the proteolytic attack did not occur during purification, since the relative amount of this form did not increase during the course of preparation.

Finally the amounts of the three different forms were determined in the livers from rats that had been subjected to different diets. The enzyme form believed to be proteolytically modified was most abundant in livers from starved animals, where it accounted for about 15 % of the total activity. The corresponding values for the group of rats fed *ad libitum* was 10 %, while it was 5 % for those held on a fructose-rich diet. The phosphorylated form of pyruvate kinase was predominant in all diet groups, amounting to between one half and two thirds of the total activity.

FRUCTOSE-1,6-BISPHOSPHATASE

Fructose-1,6-bisphosphatase (E. C. 3. 1. 3. 11; Fru-1,6-P2:ase) catalyzes the hydrolysis of D-fructose-1,6-bisphosphate to D-fructose 6-P. Fru-1,6-P2:ase was first described in 1943 (34) as a Mg^{2+} requiring alkaline phosphatase found in liver and kidney tissue.

Fru-1,6-P2:ase forms part of a control point in the glycolytic and gluconeogenetic pathways. A close regulation of the enzyme is important since the simultaneous operation of both Fru-1,6-P2:ase and phosphofructokinase within a single system would lead to hydrolysis of ATP and thus to a short-circuit in metabolism (37).

Occurrence

Fru-1,6-P2:ase has been isolated and characterized from many systems e.g. mammal liver, kidney, intestine, muscle, mammary glands, brown adipose tissue, brain and placenta (for a complete review see 78). The highest activity of Fru-1,6-P2:ase is seen in gluconeogenetic tissues such as liver and kidney cortex.

Mammalian Fru-1,6-P2:ase is a tetrameric enzyme having identical subunits and having a molecular weight of about 140 000. The complete amino acid sequence has been determined for Fru-1,6-P2:ase from pig kidney cortex (48) and sheep liver (29). These sequences show a high degree of homology with 90 % being identical (36).

Native Fru-1,6-P2:ase has a neutral pH-optimum (e. g. 79). It was shown that digestion of native Fru-1,6-P2:ase with papain (61) and subtilisin (80) yielded enzyme forms with alkaline pH-optima by removal of the N-terminal part of the polypeptide chains.

The main effectors of mammalian Fru-1,6-P2:ase activity

Fru-1,6-P2:ase has an absolute requirement for a divalent cation such as Mg^{2+} (34), Mn^{2+} (52) or Co^{2+} (43), but it is inhibited by higher concentrations of Mg^{2+} (76).

Zn^{2+} has a dual role concerning the activity of Fru-1,6-P2:ase; it inhibits the activity at low concentrations, while at higher concentrations it can replace Mg^{2+} or Mn^{2+} as the activating cation (58).

Ca^{2+} in concentrations of a couple of hundred micromolar is a potent inhibitor of Fru-1,6-P2:ase (79).

Intact Fru-1,6-P2:ase is inhibited by AMP at millimolar levels (55, 75). Subtilisin digestion of the enzyme abolishes the inhibitory effect of AMP (61). It has also been reported that Fru-1,6-P2:ase is inhibited by ADP and ATP but higher concentrations are needed than of AMP for a similar effect (30, 51, 75).

Fru-2,6-P2 in the micromolar range is a potent inhibitor of Fru-1,6-P2:ase activity. Its effect is synergistic with that of AMP (for a review see 38). Hormonal studies have shown that the concentration of Fru-2,6-P2 in rat liver can be lowered by both glucagon and epinephrine (31, 62).

By and large, present knowledge of the molecular interactions between Fru-1,6-P2:ase and the catalytic metal ion, AMP, Fru-2,6-P2 and Fru-1,6-P2 may be summed up as follows: Binding of the metal ion and AMP is of competitive nature; binding of Fru-2,6-P2 and binding of Fru-1,6-P2 are also mutually exclusive; and binding of Fru-2,6-P2 increases the affinity of the allosteric site for AMP (68).

The activity of Fru-1,6-P2:ase is also affected by a number of activating substances such as the chelating agents EDTA, histidine and citrate (59) and nonchelating agents like fatty acids (1) and phospholipids (7).

Phosphorylation of rat liver Fru-1,6-P2:ase

It was reported in 1977 (64) that purified rat liver Fru-1,6-P2:ase is a substrate for cAMP-PK in the presence of Mg^{2+} and with (^{32}P)ATP as phosphate donor, and it was seen that phosphorylation occurs both in vitro and in vivo. About 4 mol of phosphate were incorporated per mol of enzyme tetramer and bound to serine (64). It was observed that trypsin digestion removed peptides from the enzyme containing all the incorporated phosphate (60). The amino acid sequence around the phosphorylated serine was determined (60) as Ser-Arg-Pro-Ser(P)-Leu-Pro-Leu-Pro. The same sequence except with Tyr in position 3 was found (40) and a synthetic peptide with the same sequence proved to be a substrate for cAMP-PK although with a comparatively high K_m.

The phosphorylatable amino acid sequence was shown to be located close to the C-terminal end of the enzyme subunits (39) and its exact location was determined as Ser-341 (65).

Reports of a second phosphorylated amino acid in the C-terminal sequence of Fru-1,6-P2:ase appeared when it was found that another serine residue beyond Arg-348 was phosphorylated (65). This residue was later identified as Ser-356 (8) within the sequence Ser-Arg-Ala-Arg-Glu-Ser-Pro-Val-His-Ser(P)-Ile.

The influence of phosphorylation on the activity of Fru-1,6-P2:ase has been the subject of some dispute. A few authors have not detected any change in activity (e. g. 65) while others have detected an increase in V_{max} (64), or a decrease in apparent K_m (20, 50).

The effect of fructose 2,6-bisphosphate and AMP on the activity of phosphorylated and unphosphorylated fructose-1,6-bisphosphatase from rat liver.

Rat liver Fru-1,6-P2:ase was phosphorylated with the catalytic subunit of cAMP-PK. The reaction was interrupted by removal of (^{32}P)ATP on a Sephadex G-50 column before the phosphorylation reaction was estimated to be completed. The partially phosphorylated Fru-1,6-P2:ase was subjected to chromatofocusing which yielded two fractions with enzyme activity: one with an apparent pI of about 5.0 and the other of about 4.5. The former fraction corresponded to unphosphorylated and the latter to phosphorylated Fru-1,6-P2:ase (15).

The effects of Fru-2,6-P2 and AMP on the two enzyme forms were examined and it was found that unphosphorylated Fru-1,6-P2:ase was more sensitive than the phosphorylated enzyme to both inhibitors. AMP acted by decreasing the V_{max} of the enzyme; the presence of 25 μM AMP decreased the V_{max} to 70 % of the uninhibited value for the phosphorylated and to 40 % for the unphosphorylated form of Fru-1,6-P2:ase. The effect of Fru-2,6-P2 was to lower V_{max} as well as to increase the apparent K_m for Fru-1,6-P2. The amount of Fru-2,6-P2 needed for a 50 % decrease in V_{max} was 11 μM for the phosphorylated and 4 μM for the unphosphorylated enzyme. It was also shown that the combined effect of AMP and Fru-2,6-P2 on the V_{max} of Fru-1,6-P2:ase was more than additive.

The finding that the phosphorylated and unphosphorylated forms of Fru-1,6-P2:ase differ in their sensitvity to the two important inhibitors Fru-2,6-P2 and AMP, and the observation that those inhibitors act synergistically, might provide a way to amplify the effect of phosphorylation. Administration of glucagon to hepatocytes induces phosphorylation of both Fru-1,6-P2:ase (9) and the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (21, 67). The effect of phosphorylation of the latter enzyme is an inhibition of the kinase and a stimulation of the phosphatase activity. So the administration of glucagon gives a decrease in the concentration of Fru-2,6-P2 as well as in the amount of unphosphorylated and more easily inhibited Fru-1,6-P2:ase.

These observations give rise to speculations that the physiologically most important effect of phosphorylation of Fru-1,6-P2:ase is to decrease the sensitivity to AMP and Fru-2,6-P2 rather than to affect the apparent K_m for Fru-1,6-P2.

Fructose-1,6-bisphosphatase from rat liver. A comparison of the kinetics of the unphosphorylated enzyme and the enzyme phosphorylated by cyclic AMP-dependent protein kinase.

The purification procedure for Fru-1,6-P2:ase was improved with the object to minimize the possibility of proteolytic attack which may potentially result in a product with an alkaline pH-optimum, if the N-terminal end is cleaved off (61), or alternatively may result in an enzyme impossible to phosphorylate, if the C-terminal end is removed (59). These improvements resulted in an increase in the incorporation of phosphate and an increased pH-ratio, the activity of the enzyme at pH 7.5 divided by that at pH 9.2, (16).

It was investigated whether the presence of the inhibitor Fru-2,6-P2 affected the rate of phosphorylation of Fru-1,6-P2:ase, as in the yeast enzyme (32), but no such effect could be seen.

The effects of various factors on the activity of phosphorylated and unphosphorylated Fru-1,6-P2:ase were determined. It was confirmed that unphosphorylated Fru-1,6-P2:ase was more susceptible to inhibiton by AMP and Fru-2,6-P2, while both forms were

inhibited equally but less efficiently by ADP and ATP, implying that the two former inhibitors might be more important for the regulation of Fru-1,6-P2:ase.

Both phosphorylated and unphosphorylated Fru-1,6-P2:ase was fully active at 1 mM Mg^{2+} and no inhibition was seen at concentration of up to 5 mM. The two forms also had neutral pH-optima although the profile for phosphorylated Fru-1,6-P2:ase was more level. Citrate, 3 mM, or histidine, 1 mM, did not affect the activity of either of the forms.

Under conditions resembling the metabolic state during gluconeogenesis the phosphorylated form of Fru-1,6-P2:ase had twice the activity of the unphosphorylated.

Effects of epinephrine, glucagon and insulin on the activity and degree of phosphorylation of fructose-1,6-bisphosphatase in cultured hepatocytes.

Hepatocytes were kept in primary culture for 24 h. After that time they were incubated with or without radioactive (^{32}P)orthophosphate for 30 min to label endogenous ATP, and fresh medium containing the hormones to be tested was added. After a suitable period of incubation the medium was sucked off and the cells were frozen in liquid nitrogen (17).

The activity of Fru-1,6-P2:ase was analyzed in the thawed and centrifuged cell extract. Changes in K_m were monitored as changes in the activity at 12.5 μM Fru-1,6-P2 divided by that at 83 μM (corresponding to an approximately saturating substrate concentration).

The degree of phosphorylation of Fru-1,6-P2:ase was determined after removal of the other labeled cell components by chromatography on a column with anti-Fru-1,6-P2:ase coupled to CNBr-activated Sepharose.

It was seen that glucagon caused a decrease in apparent K_m as judged from the change of the activity ratio from 0.57 to 0.66. Maximal effect was obtained with a glucagon concentration of 2×10^{-11} M , the effect was complete within 10 min and lingered on for at least 2 hours.

Epinephrine increased the activity ratio of Fru-1,6-P2:ase even more, to a ratio of 0.76, and more quickly. The concentration of hormone needed for maximal effect was 2×10^{-5} M; activation was complete within 2-5 min and lasted for less than 15 min.

Treatment of the hepatocytes with insulin resulted in an increase in apparent K_m as judged from the decrease in the activity ratio to 0.46. The effect reached a maximum in about 10 min and had disappeared after another 10 min. An insulin concentration of 1×10^{-8} M gave maximal decrease in the activity of Fru-1,6-P2:ase.

It was shown that the effect of epinephrine on the activity of Fru-1,6-P2:ase was mediated by ß-receptors since the activation was inhibited by the presence of the ß-receptor blocking agent propanolol. No effect could be seen by phentolamine, an α-receptor antagonist. Propanolol, which acts on both $ß_1$- and $ß_2$- receptors, was able to suppress the activation by epinephrine totally at a concentration where metoprolol, an antagonist specific for $ß_2$-receptors, only diminished the activation by 50 %.

Treatment of the (^{32}P)labeled hepatocytes with glucagon increased the incorporation of phosphate from 2.5 mol per mol of tetrameric enzyme (found in Fru-1,6-P2:ase from cells which had not been exposed to hormone) to 4.2 mol per mol. Epinephrine stimulated phosphorylation to 3.5 mol of phosphate per mol of enzyme. The effect of the two hormones in this respect was additive. In contrast, insulin brought about a decrease in the degree of phosphorylation to 2.0 mol per mol enzyme tetramer.

It was also shown that the effect of glucagon and epinephrine was not synergistic since addition of the two hormones together affected the activity no more than epinephrine alone.

Rat liver fructose-1,6-bisphosphatase: Identification of serine-338 as a third major phosphorylation site for cyclic AMP-dependent protein kinase. Activity changes associated with multisite phosphorylation in vitro.

Purified rat liver Fru-1,6-P2:ase was phosphorylated with (^{32}P)ATP and the catalytic subunit of cAMP-PK. A maximum incorporation of 2.6 mol of (^{32})phosphate per enzyme subunit was reached (18).

Digestion of the phosphorylated enzyme with trypsin yielded two phosphopeptides containing 68 and 32 % of the total radioactivity, respectively. The former peptide started with amino acid No. 336 in the complete sequence and contained the sequence Ala-Lys-Ser(P)-Arg-Pro-Ser(P)-Leu-Pro. Previously Ser-341 had been reported to be a phosphorylation site for cAMP-PK (64, 65), but the observation that also Ser-338 was phosphorylated had not been made before. The second peptide starting with amino acid No. 351 contained the sequence Glu-Ser-Pro-Val-His-Ser-Ile-Cys-Asp in which one phosphorylated serine, first identified as No. 352 (65) but later as No. 356 (8) was phosphorylated. The second of these identifications was confirmed (18).

It was shown that phosphorylation of Ser-356 required the intact three-dimensional structure of the enzyme as evidenced by the observation that this site was not phosphorylatable after unfolding of the enzyme with 4 M urea prior to phosphorylation. (The urea was diluted in the phosphorylation mixture to a final concentration of 1.6 M, which had no effect on the activity of the protein kinase under the experimental conditions.) It was also impossible to phosphorylate a synthetic peptide with the same sequence at a significant rate. This might indicate that the basic amino acid(s) needed close to the phosphorylation site are located in another part of the amino acid sequence.

It was concluded that the K_m-values for phosphorylation of the three different phosphorylation sites were very similar, since all three sites were phosphorylated at the same rates in the intact enzyme. Fru-1,6-P2:ases incorporating between 0.30 and 2.60 mol (^{32}P)phosphate per mol subunit were all found to contain 1/3 of the radioactivity bound to each serine.

It was shown that peptides containing the three different phosphorylatable serine residues could be sequentially cleaved off by first incubating the enzyme with

chymotrypsin, which removed a peptide containing Ser-356, then with 4 M urea followed by a higher concentration of chymotrypsin, which removed a peptide containing Ser-341, and finally with trypsin, which digested a tripeptide containing Ser-338 (Figure 1). This stepwise progressive digestion method provided a tool for investigating at which site or sites Fru-1,6-P2:ase isolated from hepatocytes is phosphorylated in response to different hormones.

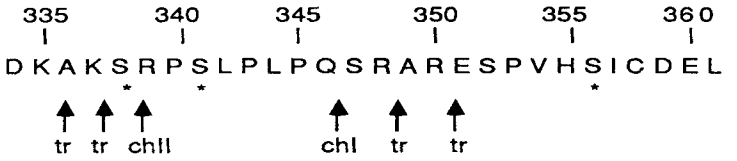

Figure 1. The amino acid sequence of the C-terminal region of rat liver Fru-1,6-P2:ase as reported in 65). The figure includes the location of the phosphorylation sites (*) as well as the sites of proteolysis by trypsin (tr) and by chymotrypsin before (ch I) and after (ch II) exposure to urea.

As seen earlier (15, 16) phosphorylated Fru-1,6-P2:ase had a lower K_m for Fru-1,6-P2 and was less readily inhibited by AMP and Fru-2,6-P2. Removal of Ser-356 did not affect any of these parameters. When Ser-341 was removed both phosphorylated and unphosphorylated Fru-1,6-P2:ase were affected equally by both inhibitors, but the phosphorylated enzyme still had a lower K_m for Fru-1,6-P2.

In vitro phosphorylation of fructose-1,6-bisphosphatase from rabbit and pig liver with cyclic AMP-dependent protein kinase.

Fru-1,6-P2:ase was purified from rat, rabbit, pig, mouse and human liver using the method described earlier (16). Fru-1,6-P2:ases from all species were tested as substrates for cAMP-PK with (^{32}P)ATP as the phosphate donor (19).After incubation they were subjected to polyacrylamide gel electrophoresis and the radioactivity was located by autoradiography. It was found that, in addition to rat liver Fru-1,6-P2:ase, the enzymes from pig and rabbit liver were phosphorylatable, both with up to 1 mol of phosphate per mol enzyme subunit. The incorporation of (^{32}P)phosphate found in Fru-1,6-P2:ase from mouse and human liver was less than 0.05 mol per mol enzyme subunit.

Phosphorylation of pig and rabbit liver Fru-1,6-P2:ase decreased the apparent K_m for Fru-1,6-P2 but, in contrast to the rat liver enzyme, had no effect on the degree of

inhibition by AMP and Fru-2,6-P2.

The subunit molecular weight (Mr) for rat liver Fru-1,6-P:ase was 41 kd. For all other species investigated it was 37 kd. It has earlier been reported that trypsin digestion of phosphorylated Fru-1,6-P2:ase removes peptides containing all of the phosphorylated serine residues from the C-terminal of the polypeptide chain (60), leaving a core protein with Mr 37 kd. No decrease in molecular size could be seen after trypsin digestion of Fru-1,6-P2:ase from any of the sources except rat liver. However, digestion of (^{32}P)-labeled pig and rabbit liver Fru-1,6-P2:ase removed the protein-bound radioactivity quantitatively.

These observations suggest that Fru-1,6-P2:ase from pig and rabbit liver is phosphorylated on at least one amino acid residue very close to the C-terminal end of the enzyme subunits. So it would seem that regulation of the activity of Fru-1,6-P2:ase by reversible phosphorylation is not a feature unique to the rat liver enzyme.

GENERAL DISCUSSION

Studies concerning the phosphorylation state of rat liver pyruvate kinase in vivo used to give somewhat contradictory results. Pyruvate kinase in hepatocytes isolated from fed rats has been reported to be phosphorylated and inactive (4) while other studies showed that pyruvate kinase in whole livers from rats fed an enriched sucrose diet was inhibited and phosphorylated with 2-3 mol phosphate/mol tetrameric enzyme (22). The latter report is compatible with the results in (14) where it is seen that phosphorylated pyruvate kinase is the predominant form in livers from rats irrespective of diet.

These discrepancies were later explained by the observation that pyruvate kinase of the L-type is dephosphorylated during perfusion of the liver, as demonstrated by a change in the apparent K_m and by increased phosphorylatability of the enzyme after perfusion (63).

Earlier studies have shown that phosphorylated pyruvate kinase is more prone to proteolytic degradation than the unphosphorylated enzyme in vitro (3). The detection of a form of pyruvate kinase in vivo, which has been degraded proteolytically but still has enzymatic activity may indicate that phosphorylation of pyruvate kinase not only regulates the activity but also initiates degradation of the enzyme.

It was recently shown that pyruvate kinase was phosphorylated in vitro with 1.7 mol phosphate/mol enzyme subunit by a Ca^{2+}-calmodulin dependent protein kinase (69). This caused an increase in apparent K_m which was greater than that brought about by phosphorylation with cAMP-PK. It was also shown that one site phosphorylated by Ca^{2+}-calmodulin activated protein kinase was identical to that phosphorylated by cAMP-PK. This report invites speculations about the intriguing nature of hormone interaction in the process of pyruvate kinase regulation, in particular the interaction between glucagon, which acts via cAMP-PK, and hormones such as epinephrine, which

affect the Ca^{2+} flux to thus activate Ca^{2+}-calmodulin activated protein kinase.

Phosphorylation of rat liver Fru-1,6-P2:ase, probably at more than one site, makes the enzyme less sensitive to inhibition by AMP and Fru-2,6-P2. This difference between phosphorylated and unphosphorylated Fru-1,6-P2:ase is enhanced since the two inhibitors act synergistically (e.g. 38), giving an overall activating effect on Fru-1,6-P2:ase by phosphorylation due both to a decreased apparent K_m and the decrease in sensitivity to the inhibitors. The effect of glucagon on the activity of Fru-1,6-P2:ase in the cell is further amplified by the cAMP-PK-dependent phosphorylation of the enzyme responsible for Fru-2,6-P2 homeostasis, which is conducive to a decrease in concentration (21, 62).

The identification of a third phosphorylation site for cAMP-PK in the C-terminal region of rat liver Fru-1,6-P2:ase immediately gives rise to speculations whether the enzyme is phosphorylated at different sites in response to different hormones (18). Speculations of this nature started when the second phosphorylation site was discovered (8, 65) but no conclusive evidence was given. Such an explanation might furnish an explanation of the data concerning Fru-1,6-P2 :ase form hepatocytes (17) where it is seen that both epinephrine and glucagon increase the activity and degree of phosphorylation of Fru-1,6-P2:ase but in different ways. This hypothesis is further supported by the observation that the increases in phosphorylation caused by epinephrine and glucagon are additive. The method of sequentially digesting peptides containing the phosphorylatable serine residues is a suitable tool for such studies.

One of the effects of insulin is to lower the degree of phosphorylation, accompanied by a decrease in activity of Fru-1,6-P2:ase. An explanation of this effect could be the activation of a phosphoprotein phosphatase which dephosphorylates Fru-1,6-P2:ase. The decrease in activity is not thought to be a consequence of the decrease in the level of cAMP (28).

Another question that might be raised by the occurrence of three different phosphorylation sites is whether phosphorylation of all the sites occurs in vivo. The simultaneous phosphorylation in vitro of three different sites could be an artefact due to dephosphorylation of the enzyme during the course of preparation, most probably before the heating step when most other cellular proteins are denatured.

It was observed that native Fru-1,6-P2:ases from pig and rabbit liver were phosphorylated by cAMP-PK in vitro, with a concomittant change in apparent K_m for Fru-1,6-P2 (19). This would mean that regulation of Fru-1,6-P2:ase activity by phosphorylation is not a feature unique for the rat liver enzyme. However in contrast to the rat liver enzyme, phosphorylation of Fru-1,6-P2:ase from pig and rabbit had no effect on the inhibition by AMP and Fru-2,6-P2.

The capacity of glucagon and related hormones is to stimulate cAMP-PK to phosphorylate among many other proteins pyruvate kinase, Fru-1,6-P2:ase and 6-phosphofructo-

2-kinase/fructose-2,6-bisphosphatase. These enzymatic modifications lead to a decrease in the rate of glycolysis by inactivating pyruvate kinase and to an increase in the rate of gluconeogenesis by activating Fru-1,6-P2:ase and decreasing the amount of the inhibitor Fru-2,6-P2. The overall effect of glucagon and other hormones related to starvation is to induce gluconeogenesis and thus to maintain the glucose homeostasis.

ACKNOWLEDGEMENTS

The work by the author included in this review was financially supported by the Swedish Medical Research Council (grant 13X-50) and by Sven och Lilly Lawskis fond för naturvetenskaplig forskning.

REFERENCES

1. Baxter, R. C., Carlson, C. W. & Pogell, B. M.: Stimulation of the neutral activity of rabbit liver fructose-1,6-diphosphatase by fatty acids.J. Biol. Chem. 247:2969-2971, 1972.
2. Belfrage, P., Jergil, B., Strålfors, P. & Tornqvist, H.: Hormone sensitive lipase of rat adipose tissue: identification and som properties of the enzyme protein. FEBS Lett. 75:259-264, 1977.
3. Bergström, G., Ekman, P., Humble, E. & Engström, L.: Proteolytic modification of pig and rat liver pyruvate kinase type L including phosphorylatable site. Biochim. Biophys. Acta 532:259-267, 1978.
4. Blair, J. B., Cimbala, M. A. & James, M. E.: Hepatic Pyruvate kinase. Quantitative measurements of phosphorylation in vitro and in the isolated rat hepatocyte. J. Biol. Chem. 257:7595-7602, 1982.
5. Brand, I. A. & Söling, H. D.: Activation and inactivation of rat liver phosphofructokinase by phosphorylation-dephosphorylation. FEBS Lett. 57:163-168, 1975.
6. Burnett, G. & Kennedy, E. P.: The enzymatic phosphorylation of proteins. J. Biol. Chem. 211:969-980, 1954.
7. Carlson, C. W., Tejwani, G. A., Baxter, R. C., Ulm, E. H. & Pogell, B. M.: Involvement of cytosol proteins in oleate activation of rabbit liver fructose-1,6-diphosphatase. J. Biol. Chem. 250:4996-5002, 1975.
8. Chatterjee, T., Rittenhouse, J., Marcus, F., Reardon, I. & Heinrikson, R. L.: Identification of the in vivo and in vitro phosphoryation sites of rat liver fructose-1,6-bisphosphatase. J. Biol. Chem. 259:3831-3833, 1984.
9. Claus, T. H., Schlumpf, J., El-Maghrabi, M. R., McGrane, M. & Pilkis, S. J. : Glucagon stimulation of fructose-1,6-bisphosphatase in rat hepatocytes. Biochem. Biophys. Res. Commun. 100:716-723, 1981.
10. Cohen, P.: The role of protein phosphorylation i the hormonal control of enzyme activity. Eur. J. Biochem. 151:439-448, 1985.
11. Cooper, J. A. & Hunter, T.: Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. Mol. Cell. Biol. 1:165-178, 1981.
12. Dahlqvist-Edberg, U. & Ekman, P.: Purification of a Ca^{2+}-activated protease from rat erythrocytes and its possible effect on pyruvate kinase in vivo. Biochim. Biophys. Acta 660:96-101, 1981.
13. Eckhart, W., Hutchkinson, M. A. & Hunter, T. : An actitity phosphorylating tyrosine in polyoma T antigen precipitates. Cell, 18:925-933, 1979.
14. Ekdahl, K. N. & Ekman, P.: Hepatic L-type pyruvate kinase: Separation of unphosphorylated, phosphorylated and proteolytically modified in vivo forms. J Biochem. 95: 917-924, 1984.
15. Ekdahl. K. N. & Ekman, P.: The effect of fructose-2,6-bisphosphatase and AMP on the activity of phosphorylated and unphosphorylated fructose-1,6-bisphosphatase. FEBS Lett. 167:203-209, 1984.
16. Ekdahl, K. N. & Ekman, P.: Fructose-1,6-bisphosphatase from rat liver. A comparison

of the kinetics of the unphosphorylated enzyme and the enzyme phosphorylated with cyclic AMP-dependent protein kinase. J. Biol. Chem. 260:14173-14179, 1985.
17. Ekdahl, K. N. & Ekman, P.: Effects of epinephrine, glucagon and insulin on the activity and degree of phosphorylation of fructose-1,6-bisphosphatase in cultured hepatocytes. Biochim. Biophys. Acta 929:318-326, 1987.
18. Ekdahl, K. N.: Rat liver fructose-1,6-bisphosphatase: Identification of serine-338 as a third major phosphorylation site for cyclic AMP-dependent protein kinase. Activity changes associated with multisite phosphorylatio in vitro J. Biol. Chem. in press, 1987.
19. Ekdahl, K. N.: In vitro phosphorylation of fructose-1,6-bisphosphatase from rabbit and pig liver with cyclic AMP-dependent protein kinase Arch. Biochem. Biophys. in press, 1987.
20. Ekman, P., & Dahlqvist-Edberg, U.: The kinetics of unphosphorylated, phosphorylated and proteolytically modified fructose bisphosphatase from rat liver. Biochim. Biophys. Acta 662:265-270, 1981.
21. El-Maghrabi, M. R., Claus, T. H., Pilkis, J., Fox, E., & Pilkis, S. J.: Regulation of rat liver fructose-1,6-bisphosphatase J. Biol. Chem. 257:7603-7607, 1982.
22. El-Maghrabi, M. R., Haston, W. S., Flockhart, D. A., Claus, T. H. & Pilkis, S. J.: Studies on the phosphorylation and dephosphorylation of L-type pyruvate kinase by the catalytic subunit of cyclic AMP-dependent protein kinase. J. Biol. Chem. 255:668-675, 1980.
23. Engström, L.: Regulation of liver pyruvate kinase by phosphorylation-dephosphorylation. in Recently discovered systems of enzyme regulation by reversible phosphorylation (Cohen, P. ed) pp 11-31, Elsevier,Amsterdam, 1980.
24. Engström, L., Edlund, B., Ragnarsson, U., Dahlqvist-Edberg, U. & Humble, E.: Phosphorylation of human fibrinogen in vitro with cyclic 3', 5' AMP-stimulated protein kinase and (^{32}P) ATP. Biochem. Biophys. Res. Commun. 96:1503-1507, 1980.
25. Engström, L., Ekman, P., Humble, E., Ragnarsson, U. & Zetterqvist, Ö. : Detection and identification of substrates for protein kinases: use of proteins and synthetic peptides . Methods in Enzymol. 107:130-154, 1984.
26. Engström, L., Ekman, P., Humble, E. & Zetterqvist, Ö. : Pyruvate kinase. in The Enzymes vol XVIII, 3d ed. pp. 47-75, Academic Press, Inc., 1987.
27. Exton, J. H., Robinson, G. A., Sutherland, E. W. & Park, C. R.: Studies on the role of adenosine 3', 5'-monophosphate in the hepatic action of glucagon and catecholamines. J. Biol. Chem. 246,:6166-6177, 1971.
28. Felíu, J. E., Hue, L. & Hers, H. G.: Hormonal control of pyruvate kinase activity and of gluconeogenesis in isolated hepatocytes. Proc. Natl. Acad. Sci. U. S. A. 73:2762-2766, 1976.
29. Fisher, W. K. & Thompson, E. O. P.: Amino acid sequnece studies on sheep liver fructose-bisphosphatase II The compelete sequence. Aust. J. Biol. Sci. 36:235-250, 1983.
30. Fu, J. Y. & Kemp, R. G. : Activation of muscle fructose-1,6-diphosphatase by creatine phosphate and citrate. J. Biol. Chem. 248:1124-1125, 1973.
31. Furuya, E., Yokoyama, M. & Uyeda, K.: Regulation of phosphofructokinase by a new mechanism. Proc. Natl. Acad. Sci. U. S. A. 79:325-329, 1982.
32. Gancedo, J. M., Mazón, M. J. & Gancedo, C: Fructose-2,6-bisphosphate activates cAMP-dependent phosphorylation of yeast fructose-1,6-bisphosphatase in vitro. J. Biol. Chem. 258:5998-5999, 1983.
33. Glass, D. B. & Krebs, E. G.: Protein phosphorylationcatalyzed by cyclic AMP-dependent and cyclic GMP-dependent protein kinases Ann. Rev. Pharmacol. Toxicol. 20:363-388, 1980.
34. Gomori, G.: Hexosephosphatase. J. Biol. Chem. 148:139-149, 1943.
35. Guy, P. S., Cohen, P. & Hardie, D. G.: Rat mammary gland ATP-citrate lyase is phosphorylated by cyclic AMP-dependent protein kinase. FEBS Lett. 109:205-208, 1980.
36. Harrsch, P. B., Kim, Y., Fox., J. L. & Marcus, F.: Amino acid sequence similarity between spinach chloroplast and mammalian gluconeogenic fructose-1,6-bisphosphatase. Biochem. Biophys. Res. Commun. 133:520-526, 1985.
37. Hers, H. G. & Hue, L.: Gluconeogenesis and related aspects of glycolysis. Ann. Rev. Biochem. 32:617-633, 1983.

38. Hers, H. G. & Van Schaftningen, E.: Fructose-2,6-bisphosphate 2 years after its discovery. Biochem. J. 206:1-12, 1982.
39. Hosey, M. M. & Marcus, F.: Fructose-bisphosphatase as a substrate of cyclic AMP-dependent protein kinase. Proc. Natl. Acad. Sci. U. S. A. 78:91-94, 1981.
40. Humble, E., Dahlqvist-Edberg, U., Ekman, P., Netzel, E., Ragnarsson, U. & Engström, L.: Amino acid sequence at the phosphorylated site of rat liver fructose-1,6-bisphosphatase and phosphorylation of a corresponding synthetic peptide. Biochem. Biophys. Res. Commun. 90:1064-1073, 1979.
41. Huttunen, J. K., Steinberg, D. & Mayer, S. E.: Protein kinase activation and phosphorylation of a purified hormone-sensitive lipase. Biochem. Biophys. Res. Commun. 41:1350-1356, 1970.
42. Kemp, B. E., Graves, D. J., Benjamini, E. & Krebs, E. G.: Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. J. Biol. Chem. 252:4888-4894, 1977.
43. Kirtley, M. E. & Dix, J. C.: Activation of fructose diphosphatase by manganese, magnesium and cobalt. Arch. Biochem. Biophys. 147:647-652, 1971.
44. Krebs, E. G.: The mechanism of hormonal regulation by cyclic AMP. Endocrinology, Proc. 4th Int. Congr. (Scow, R O. ed.) pp. 17-29, Excerpta Medica, Amsterdam, 1973.
45. Krebs, E. G. & Beavo, J. A.: Phosphorylation-dephosphorylation of enzymes. Ann. Rev. Biochem. 48:923-959, 1979.
46. Langan, T. A.: Histone phosphorylation: stimulation by 3', 5'-monophosphate. Science 162:579-580, 1968.
47. Ljungström, O., Hjelmqvist, G. & Engström, L: Cyclic 3', 5'-AMP-stimulated and non-stimulated phosphorylation of protein fractions from rat-liver cell sap on icubation with (γ ^{32}P)ATP. Biochim. Biophys.Acta 358:289-298 , 1974.
48. Marcus, F., Edelstein, I, Reardon, I. & Heinrikson, R. L.: Complete amino acid sequence of pig kidney fructose-1,6-bisphosphatase Proc. Natl. Acad. Sci. U.S.A. 79:7161-7165, 1982.
49. Marie, J., Tichonicky, L., Dreyfus, J. C. & Kahn, A.: Endogeneous, cyclic 3', 5'-AMPdependent phosphorylation of human red cell pyruvate kinase Biochem. Biophys. Res. Commun. 87.862-868, 1979.
50. Meek, D. W. & Nimmo, H. G.: Effects of phosphorylation on the kinetic proteprties of rat liver fructose-1,6-bisphosphatase. Biochem. J. 222:125-130, 1984.
51. Mendicino, J., Beaudreau, C. & Bhattacharyya, R. N.: Reversible inactivation of D-fructose-1,6-diphosphatase by adenosine triphosphate and 3', 5'-adenosine monophosphate. Arch. Biochem. Biophys. 116: 436-445, 1966.
52. Mokrasch, L. C. & McGlivery, R. W.: Purification and properties of fructose-1,6-diphosphatase J. Biol. Chem. 221:909-917, 1956.
53. Mårdh, S.: Phosphorylation of a kidney preparation of Na,K-ATP:ase by the catalytic subunnit of cAMP-dependent protein kinase. Curr. Top. Membranes Transport. 19:999-1004, 1983.
54. Nagano, M., Ishibashi, H., McCully, V. & Cottam, G. L.: Epinephrine stimulated phopsphorylation of pyruvate kinase in hepatocytes. Arch. Biochem. Biophys. 203:271-281, 1980.
55. Newsholme, E. A.: Some properties of fructose-1,6-diphosphatase of rat liver. Biochem. J. 89:38P, 1963.
56. Newsholme, E. A. & Start, C. (1976) in "Regulation in Metabolism" , John Wiley & Sons, Ltd, London, pp. 1-50, 1976.
57. Nimmo, H. G. & Cohen, P.: Hormonal control of protein phosphorylation. in Advances in Cyclic Nucleotide Research, Vol. 8, pp. 145-266, (Greengard, P. and Robinson, G. A. ed.) Raven, New York, 1977.
58. Nimmo, H. G. & Tipton, K. F.: Purification of fructose-1,6-diphosphatase from ox liver and its activation by ethylenediaminetetra-actetate. Biochem. J. 145:323-334, 1975.
59. Pedrosa, F. O., Pontremoli, S. & Horecker, B. L. : Binding of Zn^{2+} to rat liver fructose-1,6-bisphosphatase Proc. Natl. Acad. Sci., U. S. A. 74:2742-2745, 1977.
60. Pilkis, S. J., El-Maghrabi, M. R., Coven, B., Claus, T. H., Tager, H. S., Steiner,D. F., Keim, P. S. & Heinrikson, R. L.: Phosphorylation of rat hepatic fructose-1,6-bisphosphatase and pyruvate kinase. J. Biol. Chem. 255:2770-2775, 1980.
61. Pontremoli, S., Melloni, E. & Traniello, S.: Conversion of "neutral" to "alkaline"

fructose 1,6-diphosphatase by controlled digestion with papain. Arch. Biochem. Biophys.147:762-766, 1971.
62. Richards, C. S. & Uyeda, K.: Hormonal regulation of Fructose-6-P,2-kinase and fructose-2,6-P_2 by two mechanisms. J. Biol. Chem. 257:8854-8861, 1982.
63. Riou, J. P., Audigier, C., La Ville, M., Beylot, M., Pigeon, P. & Mornex, R.: Dephosphorylation of L-pyruvate kinase during hepatocyte isolation Arch. Biochem. Biophys. 236:321-327, 1985.
64. Riou, J. P., Claus, T. H., Flockhart, D. A., Corbin, J. D. & Pilkis, S. J.: In vivo and in vitro phosphorylation of rat liver fructose-1,6-bisphosphatase. Proc. Natl. Acad. Sci., U. S. A. 74:4615-4619, 1977.
65. Rittenhouse, J., Chatterjee, T., Marcus, F., Reardon, I. & Heinrikson, R. L.: Amino acid sequence of the COOH-terminal region of fructose-1,6-bisphosphatase in relation to cyclic AMP-dependent phosphorylation. J. Biol. Chem. 258:7648-7652, 1983.
66. Rosell-Perez, M. & Larner, J.: Studies on UDPG-alpha-glucan transglucosylase. IV Purification and characterization of two forms from rabbit skeletal muscle. Biochemistry, 3:81-88, 1964.
67. Sakakibara, R., Kitajima, S. & Uyeda, K.: Differences in kinetic protperties of phospho and dephospho forms of fructose-6-phosphate,2-kinase and fructose-2,6-bisphosphatase. J. Biol. Chem. 259:41-46, 1984.
68. Scheffler, J. E. & Fromm, H. J.: Regulation of rabbit liver fructose-1,6-bisphosphatase by metals, nucleotides, and fructose 2,6-bisphosphate as determined from fluorescence studies. Biochemistry 25:6659-6665, 1986.
69. Schworer, C. M., El-Maghrabi, M. R., Pilkis, S. J. & Soderling, T. R.: Phosphorylation of L-type pyruvate kinase by a Ca^{2+}/calmodulin-dependent protein kinase. J. Biol. Chem. 260:13018-13022, 1985.
70. Sherline, P., Lynch, A. & Glinsmann, W. H.: Cyclic AMP and adrenergic receptor control of rat liver glycogen metabolism. Endocrinology 91:680-690, 1972.
71. Shlyapnikov, S. V., Arutyunyan, A. A., Kurochkin, S. N., Memelova, L. V., Nesterova, M. V., Saschhenko, L. P., & Severin, E. S.: Investigation of the sites phosphorylated in lysine-rich histones by protein kinasea from pig brain. FEBS Lett 53:316-319, 1975.
72. Soderling, T. R., Hickenbottom, J. P., Reichmann, E. M., Hunkeler, F. L., Walsh, D. A. & Krebs, E. G. : Inactivation of glycogen synthethase and activation of phosphorylase kinase by muscle adenosine 3', 5'-monophosphate-dependent protein kinases. J. Biol. Chem. 245:6317-6328, 1970.
73. Sutherland, E. W., Jr. & Wosilait, W. D.: Inactivation and activation of liver phosphorylase. Nature 175:169-170, 1955.
74. Takai, Y., Kishimoto, A., Iwasa, Y., Kawashara, Y., Mori, T. & Nishizuka, Y. : Calcium-dependent activation of a multifunctional protein-kinase by membrane phospholipids.J. Biol. Chem. 254:3692-3695, 1979.
75. Taketa, K. & Pogell, B. M.: Reversible inactivation and inhibition of liver fructose-1,6-diphosphatase by adenosine nucleotides. Biochem. Biophys. Res. Commun. 12:229-235, 1963.
76. Tashima, Y. & Yoshimura, N.: Control of rabbit liver fructose-1,6-diphosphatase activity by magnesium ions. J. Biochem. 78:1161-1169, 1975.
77. Taunton, O. D., Stiefel, F. B., Greene, H. L. & Herman, R. H.: Rapid reciprocal changes of hepatic glycolytic enzymes and fructose-1,6-diphosphatase following glucagon and insulin injection in vivo. Biochem. Biophys. Res.Commun. 48:1663-1670, 1972.
78. Tejwani, G. A.: Regulation of fructose-bisphosphatase activity. Adv. Enzymol. 54:121-194, 1983.
79. Tejwani, G. A., Pedrosa, F. O., Pontremoli, S. & Horecker, B. L.: The purification and properties of rat liver fructose-1,6-bisphosphatase. Arch. Biochem. Biophys. 177:255-264, 1976.
80. Traniello, S. : Fructose-1,6-diphosphatase from rat liver: Purification and properties. Biochim. Biophys. Acta 341:129-137, 1974.
81. Walsh, D. A., Perkins, J. P. & Krebs, E. G.: An adenosine 3', 5'-monophosphate-dependent protein kinase from rabbit skeletal muscle. J. Biol. Chem. 243:3763-3765, 1968.
82. Zetterqvist, Ö. & Ragnarsson, U.: The structural requirements of substrates of cyclic AMP-dependent protein kinase. FEBS Lett., 139:287-290, 1982.

83. Zetterqvist, Ö., Ragnarsson, U., Humble, E., Berglund, L. & Engström, L.: The minimum substrate of cyclic AMP-stimulated protein kinase, as studied by synthetic peptides representing the phosphorylatable site of pyruvate kinase (type L) of rat liver. Biochem. Biophys. Res. Commun. 70:696-703, 1976.

Adress for correspondence:

Kristina Nilsson Ekdahl
Department of Medical and Physiological Chemistry
Biomedicum
Box 575
S-751 23 Uppsala
Sweden