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Comparative Studies on Physical Properties Concerned with the Stability of Unphosphorylated, Phosphorylated and Proteolytically Modified L-type Pyruvate Kinase from Pig Liver

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A study was made of the effects of some agents on the stability of unphosphorylated pyruvate kinase type L, the enzyme phosphorylated with cyclic 3′,5′-AMP-stimulated protein kinase and the subtilisin-modified enzyme form from which the phosphorylatable site had been removed.

The phosphorylated pyruvate kinase was found to be the most labile of the enzyme forms at high temperature and in the presence of urea. The circular dichroism spectrum of the phosphorylated enzyme also differed from that of the unphosphorylated and proteolytically modified forms. All three forms of the enzyme showed a high degree of stability over a wide pH range. The unphosphorylated enzyme seemed, however, to be the most sensitive to differences in pH. Only 10 % of its maximal activity remained after incubation at pH 10 and 30 °C for 30 min, compared with 30 % and 75 % for the phosphorylated and proteolytically modified forms of the enzyme, respectively. Of the three enzyme forms tested the subtilisin-modified pyruvate kinase was most rapidly inactivated by trypsin.

These results taken together suggest that the phosphorylated enzyme has a less ordered structure than the other two enzyme forms studied.

Liver pyruvate kinase type L (ATP: pyruvate phosphotransferase EC 2.7.1.40) is an allosteric enzyme, the activity of which is inhibited by physiological concentrations of ATP and alanine. This inhibition can be counteracted by fructose-1,6-diphosphate added in presumed physiological concentrations.[1] L-type pyruvate kinase from the pig is a tetrameric protein with subunits of equal size. The native molecular weight of the holoenzyme is about 200 000.[2]

Hepatic pyruvate kinase from the rat[3] and pig[4] are known to be substrates of cyclic AMP-stimulated protein kinase. The phosphorylated enzyme has a lower affinity for its substrate phosphoenolpyruvate, PEP, than the unphosphorylated form.[5]

When unphosphorylated and phosphorylated pyruvate kinase type L are subjected to mild proteolysis, they lose their phosphorylatable and phosphorylated sites, respectively, without any noticeable change in molecular weight on polyacrylamide gel electrophoresis in sodium dodecylsulfate.[6] To obtain the same rate of modification the unphosphorylated enzyme has to be treated with 10 times as much subtilisin as the phosphorylated form. The pyruvate kinase digested in this way still retained its full activity at saturating concentrations of PEP, but at subsaturating PEP concentrations its activity was even lower than that of the phosphorylated enzyme.[6]

Of the three forms of liver pyruvate kinase mentioned above, the unphosphorylated and the phosphorylated forms are known to exist *in vivo*,[7] and there is strong evidence to suggest that the proteolytically modified form is also present *in vivo*.[8,9] Since the three enzyme forms differ in structure and since they have different K_m for their substrate PEP, it may be assumed that the conformations of the enzymes are dissimilar. It was therefore of interest to study the effect of

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some agents on their stability, especially since the biophysical stability of proteins *in vitro* is often correlated to their stability *in vivo*.[10] Thus, the stability *in vitro* of the three forms of pyruvate kinase might possibly reflect the susceptibility of the enzyme to degradation and inhibition in the cell.

MATERIALS AND METHODS

Fructose-1,6-diphosphate, ATP, cyclic AMP, dithiothreitol, and subtilisin (subtilopeptidase A=subtilisin Carlsberg, protease type P 5380) were purchased from Sigma. Trypsin-TPCK was obtained from Worthington. (γ-^{32}P)ATP was a product of New England Nuclear Corp., Boston. DEAE-cellulose (DE-52) and phosphorylcellulose (P-11) were bought from Whatman. Sephadex gels were from Pharmacia and the hydroxylapatite from Bio-Rad Laboratories. All other chemicals were of reagent grade. Unless otherwise stated the temperature was 0–4 °C.

Pyruvate kinase type L was purified from pig liver essentially by the method described earlier.[4] The exceptions in the procedure were as follows. At the first phosphocellulose step the ion exchanger was added batchwise to the combined pyruvate kinase from the DEAE-cellulose chromatography before the phosphocellulose was packed in a column. The enzyme was eluted with a linear gradient from 20–600 mM potassium phosphate buffer, pH 5.5/30 % glycerol/0.1 mM dithiothreitol. The total gradient volume was 800 ml. The pyruvate kinase was eluted from the hydroxylapatite column with a linear gradient of totally 100 ml from 1–50 mM potassium phosphate buffer, pH 7.0. Both buffers contained 30 % glycerol, 0.1 mM dithiothreitol and 0.1 mM fructose-1,6-diphosphate.

A Zeiss PMQII spectrophotometer was used for protein determinations, using $A^{0.1\,\%}_{280\,nm}=0.68$ for the enzyme.[2] Cyclic AMP-stimulated protein kinase from rat liver was isolated through the hydroxylapatite step as described previously.[11] The specific activity of the protein kinase was 6400 units/mg. One unit of protein kinase is defined as the amount of enzyme that catalyzes the transfer of 1 pmol (^{32}P)phosphate from (^{32}P)ATP to histone 2A per min.

Pyruvate kinase (320 units/ml) was phosphorylated for 120 min at 30 °C with 1 mM (^{32}P)ATP (spec.act. 44 000 cpm/nmol), 0.01 mM cyclic AMP, 10 mM magnesium acetate and cyclic AMP-stimulated protein kinase (2240 units/ml), in 40 mM potassium phosphate buffer, pH 7.0, containing 22.5 % glycerol, 0.075 mM dithiothreitol and 0.075 mM fructose-1,6-diphosphate. The total volume was 10 ml. One unit of pyruvate kinase is defined as the amount of enzyme that catalyzes the transfer of 1 μmol phosphate from phosphoenolpyruvate to ADP per min. The incubation was interrupted by the removal of excess (^{32}P)ATP by chromatography at 4 °C on a Sephadex G-50 column (2×16 cm) equilibrated and eluted with 20 mM potassium phosphate buffer, pH 7.0/30 % glycerol/0.1 mM dithiothreitol/0.1 mM fructose-1,6-diphosphate. In order to separate the protein kinase from the phosphorylated pyruvate kinase, the combined enzyme fractions from Sephadex G-50 were applied to a 6 ml column of hydroxylapatite equilibrated and eluted with 20 mM potassium phosphate buffer, pH 7.0/30 % glycerol/0.1 mM dithiothreitol and 0.1 mM fructose-1,6-diphosphate. The phosphorylated pyruvate kinase was not retained in the column. Radioactivity was measured as Čerenkov radiation, as described by Mårdh.[12] The degree of phosphorylation was estimated to be about 0.8 mol (^{32}P)phosphate/mol subunit of pyruvate kinase in the combined fractions eluted from the hydroxylapatite column.

To remove the phosphorylated site of pyruvate kinase, 1440 units of phosphorylated enzyme in 50 mM potassium phosphate buffer, pH 7.0/15 % glycerol and 0.1 mM dithiothreitol was incubated at 20 °C with 1 ml of a freshly prepared subtilisin solution (80 μg/ml in 10 mM potassium phosphate buffer, pH 7.0). The incubation volume was 13 ml. The reaction was terminated after 30 min by passing the incubation mixture through a Sephadex G-100 column (1.6 cm×20 cm) in equilibrium and eluted with a 40 mM potassium phosphate buffer, pH 7.0/30 % glycerol/0.1 mM dithiothreitol. Of the starting material, 95 % was found to be proteolytically modified.

The method of Kimberg and Yielding[13] for the assay of pyruvate kinase was used with some modifications[3] during the purification procedure, the phosphorylation of pyruvate kinase and the subtilisin treatment of the phosphorylated enzyme. In the stability studies the pyruvate kinase activity was tested with the coupled enzyme assay of Ekman *et al.*[5] Each activity test comprised 0.02

unit of the different enzyme forms and the concentration of PEP was 5 mM. Prior to the stability tests and circular dichroism (CD) measurements, all enzyme forms were dialyzed against a 20 mM potassium phosphate buffer, pH 7.0/30 % glycerol/0.1 mM dithiothreitol.

In the tests of thermal stability, 100 μl of samples containing unphosphorylated, phosphorylated and proteolytically modified pyruvate kinase, respectively, all in a concentration of 1 unit/ml, were incubated in a water bath at different temperatures for 5, 10, 20 and 30 min. The temperature never deviated by more than ±0.5 °C from that stated. Identically composed control samples were incubated for the same length of time on ice.

The sensitivity to urea was tested by incubation of 50 μl samples, containing the three different forms of pyruvate kinase (5 units/ml) with 50 μl of urea of varying concentrations. After 15 min at 0 °C, 150 μl of 20 mM potassium phosphate buffer, pH 7.5/0.1 mM dithiothreitol/25 mM potassium chloride/0.2 % bovine serum albumin (Buffer A) were added to reduce the urea concentration. Control samples of pyruvate kinase were prepared by adding 50 μl of water instead of urea. These were incubated in the same way as the test samples.

To study the stability of the pyruvate kinase forms at different pH, acetic acid and potassium carbonate were added to achieve the pH values below and above 7.40, respectively. 25 μl of acetic acid or potassium carbonate were added to 50 μl of the enzyme. The mixtures were incubated at 30 °C for 30 min. Then 25 μl of Tris and potassium hydrogen phosphate, respectively, were thoroughly mixed with the sample to neutralize pH. Finally, 150 μl of buffer A was added to give a pyruvate kinase concentration of 1 unit/ml before the activity was tested. To the control enzyme samples water was added instead of the above-mentioned additives before the dilution with buffer A.

The proteolytic sensitivity was investigated with trypsin at different concentrations. 12.5 μl of trypsin dissolved in 10 mM hydrochloric acid were added to 50 μl (containing 1 unit/ml) of unphosphorylated, phosphorylated or subtilisin-modified pyruvate kinase, respectively, and the mixture was incubated in a water bath at 24 °C. After 20 min the pyruvate kinase activity was tested.

A Jasco J 41A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) was used for the CD measurements. The experiments were performed at room temperature (22±2 °C) in a cell with a path-length of 0.1 cm. The enzyme concentrations were between 0.068 and 0.084 mg/ml in all spectra. The mean residue weight used (w=109) was calculated from the amino acid composition reported by Kutzbach et al.[2] All measurements were repeated three times or more.

RESULTS AND DISCUSSION

The thermal effect on unphosphorylated, phosphorylated and proteolytically modified pyruvate kinase type L at 30 to 65 °C was investigated with an incubation time of 5 min (Fig. 1). All enzyme

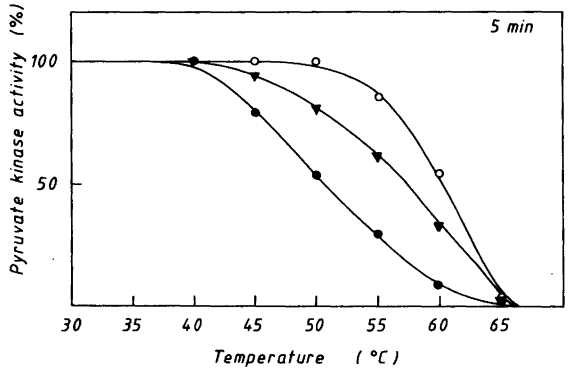

Fig. 1. (A) Thermal inactivation of unphosphorylated (○), phosphorylated (●) and subtilisin-modified (▼) pyruvate kinase type L from pig liver during five min at different temperatures.

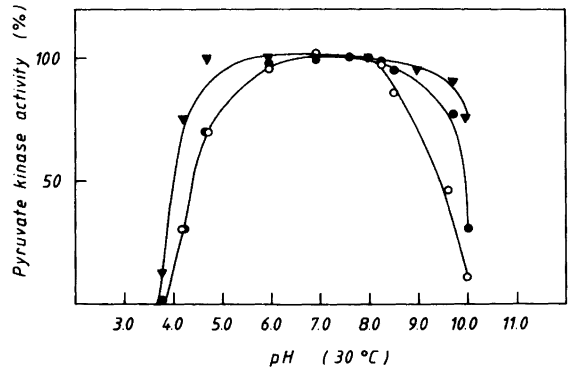

Fig. 2. The pH-stability of unphosphorylated (○), phosphorylated (●) and subtilisin-modified (▼) pyruvate kinase type L, when incubated for 30 min at 30 °C.

forms lost their activities at 65 °C. The temperatures at which half-maximal activity was noted were 51, 58 and 60 °C for the phosphorylated, subtilisin-modified and unphosphorylated enzyme forms, respectively. The difference between the enzyme forms was most pronounced at 50 °C with a 20 % discrepancy in activity between each of them. When the incubation times were varied at 50 °C, or when the incubation time was 20 min at different temperatures, the curves for the unphosphorylated and the proteolytically modified pyruvate kinase approached each other as the inactivation increased, while the curve of the phosphoenzyme was still more separated from the other two (data not shown).

The stability of the three forms of pyruvate kinase at various pH values was also investigated. The enzyme forms were very stable in this respect. When they were incubated for 30 min at 0 °C in the same pH region as seen in Fig. 2, hardly any decrease in activity was observed (not shown). When the temperature was increased to 30 °C, however, some differences between the enzyme forms were noted. The subtilisin-modified enzyme was found to be most stable against variations in pH. At pH 3.8 all three enzyme forms became completely inactivated (Fig. 2).

Below pH 7.0 the three studied forms of pyruvate kinase showed about the same stability against changes in pH. The subtilisin-modified enzyme was, however, somewhat more stable in the acidic region than the others, the difference being greatest (40 %) at a pH of about 4.0–4.5.

On the alkaline side the highest pH tested was 10.0 at 30 °C. The proteolytically modified enzyme still showed as much as 75 % of its maximal activity after incubation at this pH, the phosphorylated pyruvate kinase 30 %, and the unphosphorylated enzyme only 10 %. The pH optimum for stability of all three enzyme forms was between 6 and 8 in this study.

In order to investigate the sensitivity to further proteolysis, unphosphorylated, phosphorylated and proteolytically modified pyruvate kinase were incubated with trypsin (0–1000 μg/ml). All three pyruvate kinase forms retained about 10 % of their activity even after exposure to 1000 μg/ml of trypsin (Fig. 3). The already proteolytically modified pyruvate kinase was most sensitive to proteolysis with trypsin, as expected since more

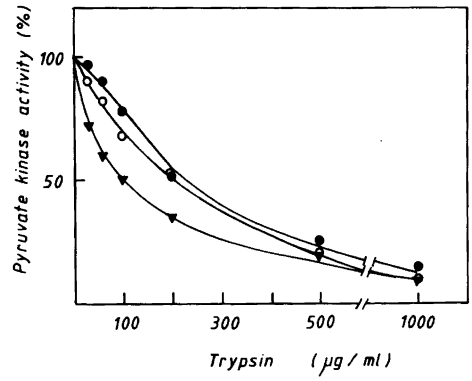

Fig. 3. The activity of unphosphorylated (○), phosphorylated (●) and subtilisin-modified (▼) pyruvate kinase type L from pig liver as a function of increasing concentrations of trypsin. The incubations were performed at 24 °C for 20 min.

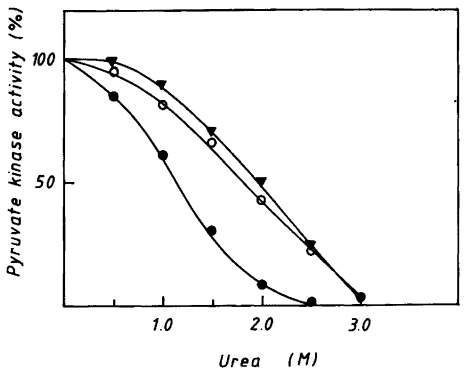

Fig. 4. The effect of urea on unphosphorylated (○), phosphorylated (●) and subtilisin-modified (▼) pyruvate kinase type L. The incubation time was 15 min at 0 °C and the urea concentrations were as indicated.

peptide bonds susceptible to cleavage are exposed after removal of the phosphorylatable peptide. A 50 % inactivation was achieved with 200 μg/ml of trypsin for the unphosphorylated and the phosphorylated enzyme, while only 100 μg/ml was required for the modified enzyme.

Denaturation of the enzyme forms in urea was tested with up to 3.0 M urea at 0 °C for 15 min.

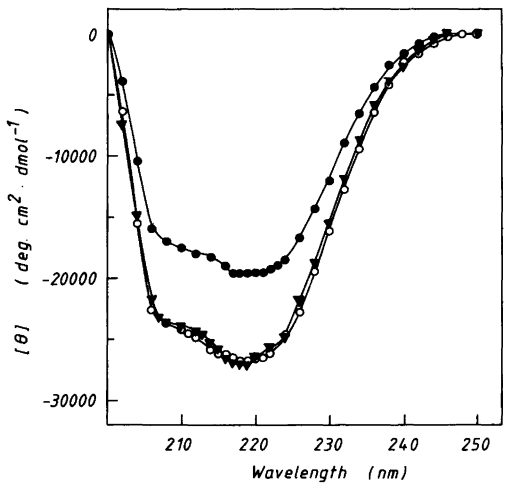

Fig. 5. Circular dichroism spectra of unphosphorylated (○), phosphorylated (●) and subtilisin-modified (▼) pyruvate kinase type L from pig liver, in 20 mM potassium phosphate buffer, pH 7.0–30 % glycerol–0.1 mM dithiothreitol at 22 °C.

At this concentration the activity of all three pyruvate kinase forms was abolished. The phosphoenzyme was the enzyme form that showed the highest sensitivity to urea. At 1.2 M urea it lost 50 % of its activity. The corresponding urea concentration giving half maximal activity for the unphosphorylated enzyme was 1.9 M, and for the subtilisin-modified form 2.0 M (Fig. 4).

Far ultraviolet CD spectra of the three forms of pyruvate kinase were measured at pH 7.0 (Fig. 5). The results indicated that the enzyme contains some α-helical conformation. The curve for the phosphorylated enzyme was of shorter descent than those for the unphosphorylated and proteolytically modified enzyme, although they all had a very similar pattern, with a distinct minimum at 219 nm. In these measurements also, the phosphorylated pyruvate kinase seemed to have characteristics in its tertiary structure which distinguished it from the other two enzyme forms. The CD spectra indicate that the proteolytically modified pyruvate kinase is very moderately digested when the phosphorylatable site has been removed.

To summarize the results of the temperature, urea and CD studies, it was found that the structures of the unphosphorylated and proteolytically modified enzyme forms were fairly similar and that these structures were more stable to temperature and urea than the phosphorylated enzyme.

Whether or not a proteolytically modified enzyme exists *in vivo* and is the first intermediate in the degradation of pyruvate kinase is not known.[14] If so, it is thought to be further degraded in the lysosomes [15,16] and would therefore probably be more labile, at least to proteolysis, than the other two forms of L-type pyruvate kinase. In this investigation the proteolytically modified enzyme was more sensitive to trypsin than the other two enzyme forms, although in all other tests it was the most stable of the three, the phosphorylated enzyme being the most labile.

Data from studies with erythrocyte pyruvate kinase [17,18] and liver pyruvate kinase [9] from the rat indicate that pyruvate kinase type L exists as a proteolytically modified form *in vivo*, but it is difficult to test and separate the different forms. The results reported in this paper suggest one way of quantifying *in vitro* the amount of proteolytically modified pyruvate kinase in a sample. It can be measured by comparing the

activity reminaing after 30 min at 30 °C and pH 10 with that remaining after the same length of time at pH 7.

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