EXISTENCE OF HOMOLOGOUS ANTIGENIC STRUCTURES IN UNFOLDED CREATINE KINASE AND ARGININE KINASE

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Received 19 January 1976

1. Introduction

Rabbit-muscle creatine kinase (EC 2.7.3.2), dimer of molecular weight 81 000 [1,2], and lobster-muscle arginine kinase (EC 2.7.3.3), monomer of molecular weight 38 000 [3], are isofunctional enzymes that share the same nucleotidic substrates, ADP and ATP, but differ in the nature of the phosphoryl acceptor guanidino substrate, creatine and arginine, respectively.

Large structural homologies have been observed in the catalytic site region of the two enzymes [4–8], which suggest that they are genetically related [9] and that minimal essential structures have been preserved through evolution [10]. However, no immunological cross-reactions were found to occur between the native molecules [11].

In this communication, we wish to report results attesting the existence of immunological relationships between the denatured forms of the two enzymes. Performic acid oxidation was used to obtain the structural disorganization of the proteins.

2. Materials and methods

Rabbit-muscle creatine kinase [12,13], lobster-muscle arginine kinase [14] and bovine serum albumin (Miles Laboratories) were oxidized with performic acid as previously described [15], yielding oxidized creatine kinase (ox-CK), oxidized arginine kinase (ox-AK) and oxidized bovine serum albumin (ox-BSA), respectively.

The oxidized proteins were labelled with ^{125}I (3.5 mCi/ml) (C.E.A., Saclay), according to the chloramine T method [16], 0.312 mCi ^{125}I being used per 100 μg protein. Average yield was 80% and specific activity was 2.62 μCi/μg for ^{125}I-ox-CK, 2.68 μCi/μg for ^{125}I-ox-AK and 2.34 μCi/μg for ^{125}I-ox-BSA. The labelled proteins were stored at -30°C in 0.07 M barbitone buffer, pH 8.6, at a concentration of 10 μg/ml.

Trypsic hydrolysis of the oxidized proteins was performed as previously described [17], the hydrolysis being restricted to the arginyl peptide bounds by previous masking of the lysyl residues with maleic anhydride [18]. After removal of the masking maleyl groups [18], the peptides were freed from unhydrolyzed protein through two consecutive filtrations on a Sephadex G-50 column (3 × 70 cm) in 10% formic acid at 20°C, then lyophilized.

The concentration of the oxidized proteins and of the crude tryptic peptides was estimated by the microbiuret method [19].

Goat antisera to ox-CK and ox-AK were prepared as indicated [13]. Rat antisera to ox-CK and ox-AK were obtained by administrating to each animal (lots of 15 female Wistar rats for each antigen) 500 μg protein emulsified in complete Freund's adjuvant (Difco), by injection in the toe pads. 10 days later, the animals received the same mixture, by intramuscular injection in the four legs. A week later, they were bled out by cardiac puncture and the antisera were tested [20]; the most reactive antisera were pooled and stored at -30°C. Rabbit antisera to rat globulins were purchased from the Institut Pasteur (Paris).

Gel diffusion tests were effected in 1% agarose

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gels for 24–48 h at 25°C [17]. The antigen-binding capacity determinations were performed according to [21] and the competition experiments according to [22].

3. Results

3.1. *Gel diffusion tests*

Definite cross-reactions were observed between ox-CK and ox-AK when compared with rat antisera to either antigen, but the reactions were weak, requiring the use of more sensitive methods.

3.2. *Radioimmunoassays*

The capacity of ^{125}I-labelled proteins to be complexed by the anti-ox-CK and anti-ox-AK antisera is reported in fig.1. The results show that antiserum to ox-CK reacts with both ox-CK and ox-AK, the degree of cross-reactivity being about 55%. Similarly, antiserum to ox-AK binds its homologous antigen with the highest efficiency, but cross-reacts ox-CK with about 30% efficiency. None of the antisera reacts with ox-BSA, which shows that the cross-reactivity between ox-CK and ox-AK is not due to haptenic groups resulting from the treatment of the protein with the performic acid reagent.

In competition experiments (fig.2), unlabelled antigens were tested for their ability to displace ^{125}I-labelled antigens complexed with their homologous antiserum. It was found that unlabelled ox-CK and ox-AK are able to displace ^{125}I-labelled ox-CK and ox-AK from their immune complexes. In both systems, the efficiency is higher with the homologous antigen

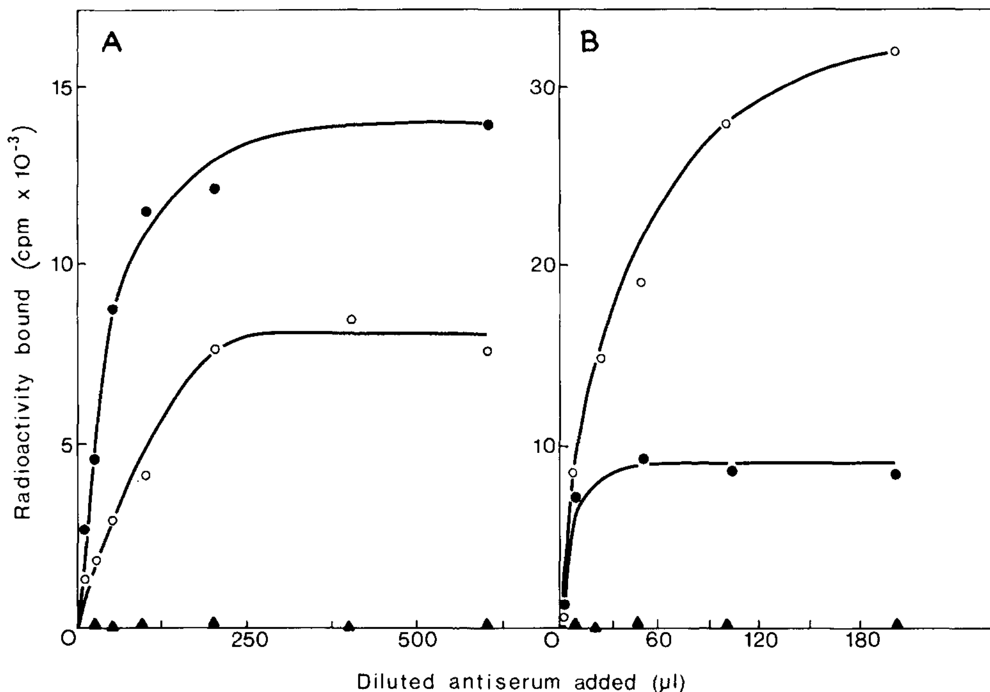

Fig.1. Antigen-binding capacity of (●–●–●) ^{125}I-labelled ox-CK (0.25 µg), (○–○–○) ^{125}I-labelled ox-AK (0.25 µg) and (▲–▲–▲) ^{125}I-labelled ox-BSA (0.25 µg) by increasing amounts of (A) rat antiserum to ox-CK (antibody dilution 1%) and (B) rat antiserum to ox-AK (antibody dilution 0.5%) in 0.15 M NaCl (total volume 1 ml for system A and 0.5 ml for system B). After 2.5 h incubation at 37°C, an excess of rabbit antiserum to rat globulins was added and the mixture was kept for 1 h at 37°C, followed by 48 h at 4°C. The precipitates were washed 3 times with 0.15 M NaCl, dissolved in M NaOH and monitored for radioactivity. Standard assays were run in the same conditions, except for the replacement of rat antisera by nonimmune rat serum, in view to determine the nonspecific radioactivity adsorbed on the antigen–antibody precipitates. The values found for the standards were deduced from those of the assays.

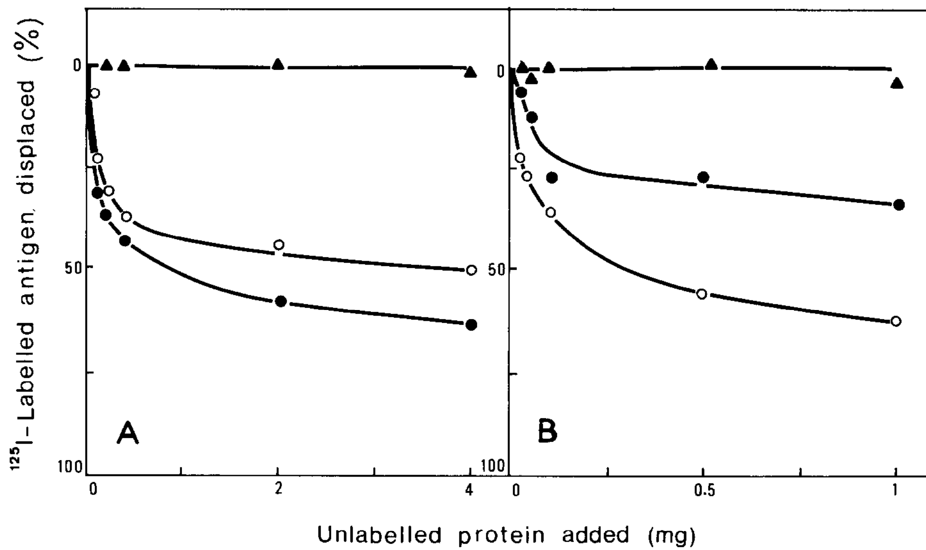

Fig.2. Displacement of ^{125}I-labelled antigens complexed with their homologous antiserum by homologous and heterologous unlabelled antigens. (A) ^{125}I-labelled ox-CK (0.6 μg) + rat antiserum to ox-CK (400 μl of a 1% dilution) and (B) ^{125}I-labelled ox-AK (0.25 μg) + rat antiserum to ox-AK (100 μl of a 0.5% dilution) were allowed to react for 1 h at 37°C in 0.15 M NaCl (total volume 0.5 ml). Increasing amounts of unlabelled proteins were added to the complexes (total volume made up with 0.15 M NaCl to 1.5 ml for system A and to 1 ml for system B): (●-●-●) ox-CK, (○-○-○) ox-AK and (▲-▲-▲) ox-BSA. After 150 min reaction at 37°C, an excess of rabbit antiserum to rat globulins was added and the assays were terminated as described in fig.1.

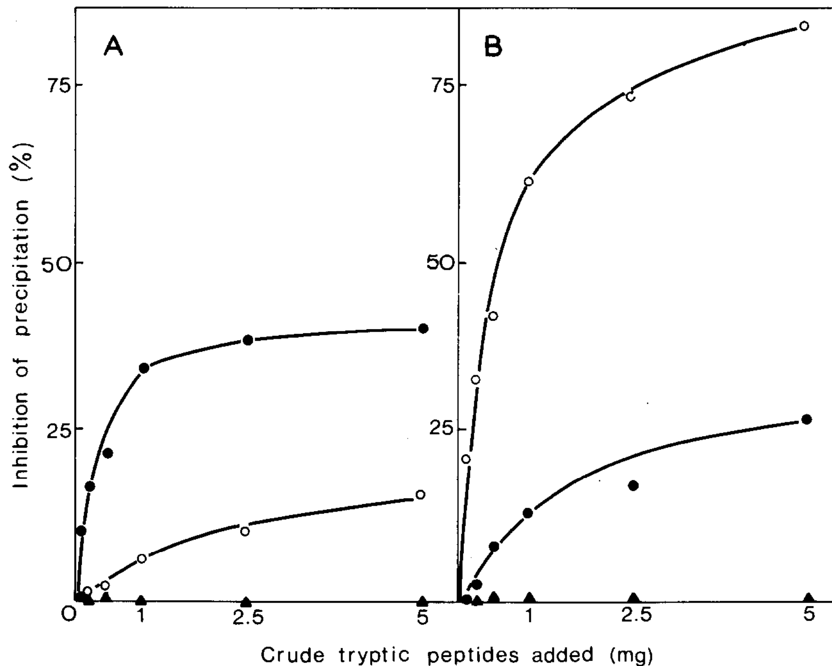

Fig.3

than with the heterologous antigen. No displacement occurs with ox-BSA.

3.3. *Inhibition studies with tryptic hydrolyzates*

The properties of tryptic hydrolyzates of the oxidized proteins to inhibit the precipitating capacity of the antisera towards their homologous antigen are shown in fig.3. The tryptic digest of ox-CK, in a protein/protein ratio of 50 over the homologous antigen, inhibits about 40% the antiserum to ox-CK and 25% the antiserum to ox-AK. In the same experimental conditions, the tryptic digest of ox-AK inhibits about 80% the antiserum to ox-AK and 15% that to ox-CK. In contrast, the tryptic digest of ox-BSA proves unable to inhibit either antiserum.

4. Discussion

Recent immunological studies concerning the lysozymes and α-lactalbumin have shown that these closely related proteins, that do not cross-react in their native form, can cross-react after chemical unfolding of their structure [21,22].

The above results were obtained using similar techniques applied to the immunological comparison of rabbit-muscle creatine kinase and lobster-muscle arginine kinase: through a series of qualitative and quantitative tests based on cross-reactions and cross-inhibitions, we have shown the existence of immunological cross-reactions between these enzymes following the strong conformational disorganization [15] caused by the performic acid oxidation.

These results establish the existence of common antigenic structures between creatine kinase and arginine kinase, that were masked in the native enzymes as a result of burrying or of immunotolerance effects. The ability of the tryptic hydrolyzates of the oxidized proteins to inhibit the antisera to the oxidized antigens suggests that the cross-reactive structures are in a large part of the sequential type.

If we consider that monomeric arginine kinase is supposed to be the most primitive form of the phosphagen kinases [9] while dimeric creatine kinase should represent the most advanced one, the existence of common antigenic structures between the two enzymes is a good argument in favour of their genetic filiation. It attests that conservative structures were preserved through the changes in the molecular size and in the substrate specificity, and confirms the homology of the vertebrate and invertebrate muscle phosphagen kinases suggested by previous biophysical and biochemical studies [10].

References

[1] Noda, L., Kuby, S. A. and Lardy, H. A. (1954) J. Biol. Chem. 209, 203–210.
[2] Bayley, P. M. and Thompson, A. R. (1967) Biochem. J. 104, 33C–35C.
[3] Regnouf, F., Kassab, R. and Fattoum, A. (1974) Eur. J. Biochem. 44, 67–79.
[4] Kassab, R., Pradel, L.-A., Der Terrossian, F. and van Thoai, N. (1967) Biochim. Biophys. Acta 132, 347–360.
[5] Pradel, L.-A. and Kassab, R. (1968) Biochim. Biophys. Acta 167, 317–325.
[6] Kassab, R., Roustan, C. and Pradel, L.-A. (1968) Biochim Biophys. Acta 167, 308–316.
[7] Roustan, C., Kassab, R., Pradel, L.-A. and van Thoai, N. (1968) Biochim. Biophys. Acta 167, 326–338.
[8] Der Terrossian, E., Pradel, L.-A., Kassab, R. and van Thoai, N. (1969) Eur. J. Biochem. 11, 482–490.
[9] Watts, D. C. (1968) in: Homologous Enzymes and Biochemical Evolution (van Thoai, N. and Roche, J. eds.), pp. 279–296, Gordon and Breach, New York.
[10] van Thoai, N. (1971) in: Prebiotic and Biochemical Evolution (Kimball, A. P. and Oró, J., eds.) pp. 172–188, North-Holland, Amsterdam.
[11] Viala, B., Robin, Y. and van Thoai, N. (1970) Comp. Biochem. Physiol. 32, 401–410.

Fig.3. Inhibitory effects of tryptic hydrolyzates of (●–●–●) ox-CK, (○–○–○) ox-AK and (▲–▲–▲) ox-BSA upon the precipitation of anti-ox-CK and anti-ox-AK antisera with their homologous antigen. (A) 1 ml of goat antiserum to ox-CK and (B) 200 μl of goat antiserum to ox-AK were incubated with increasing amounts of the crude tryptic peptides for 2 h at 37°C, followed by 15 h at 4°C, in 0.15 M NaCl (total volume 1.5 ml). 100 μg of the homologous antigen in 0.1 ml of 0.15 M NaCl were then added and the mixtures were incubated for 90 min at 37°C, followed by 48 h at 4°C. The washed precipitates were dissolved in 0.1 M NaOH and the protein concentration was estimated from the absorbance at 280 nm. The results were expressed as % inhibition of precipitation.

[12] Kuby, S. A., Noda, L. and Lardy, H. A. (1954) J. Biol. Chem. 209, 191–201.
[13] Benyamin, Y. and Robin, Y. (1975) Biochimie (Paris) 57, 1215–1219.
[14] Pradel, L.-A. and Kassab, R. (1973) Compt. Rend. Acad. Sci. Paris 276, Sér. D, 2843–2846.
[15] Benyamin, Y., Guillou, Y., Desormeau-Bedot, J.-P. and Robin, Y. (1976) submitted for publication to Eur. J. Biochem.
[16] Greenwood, F. C. and Hunter, W. M. (1963) Biochem. J. 89, 114–122.
[17] Benyamin, Y., Robin, Y. and van Thoai, N. (1973) Eur. J. Biochem. 37, 459–466.
[18] Butler, P. J. G. and Hartley, B. S., (1972) Methods Enzymol. 25, 191–199.
[19] Bailey, L. J. (1967) Techniques in Protein Chemistry, 2nd Edn. p. 341, Elsevier, New York.
[20] Ouchterlony, O. (1949) Acta Pathol. Microbiol. Scand. 26, 507–515.
[21] Arnon, R. and Maron, E. (1971) J. Mol. Biol. 61, 225–235.
[22] Arnheim, N., Sobel, J. and Canfield, R. (1971) J. Mol. Biol. 61, 237–250.