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Phosphorylation of Threonine and Serine Residues of Native and Partially Dephosphorylated Caseins by a Rat Liver Cyclic AMP-Insensitive Protein Kinase

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The preferential phosphorylation of threonine residues of native casein fractions by a rat liver cyclic AMP-independent protein kinase (EC 2.7.1.37) is abolished by preliminary limited dephosphorylation of the substrates, which promotes a fall in the phosphothreonine/phosphoserine ratios from values higher than 1 to much less than 0.1. This finding and the identification of the threonine residues phosphorylated support the view that the liver protein kinase affects threonine residues only when suitable serine residues, which fulfil the structural requirements for attack by the enzyme but which are not yet phosphorylated, are not available.

All the known phosphorylated residues in native caseins are characterized by the presence of an acidic group two residues to their C-terminal side, e.g. -Ser(P)-X-Glu [or Ser(P) or Asp] (Mercier *et al.*, 1971; Brignon *et al.*, 1977; Manson *et al.*, 1977); this is considered to be an obligatory requirement for the endogenous phosphorylation of caseins by mammary-gland casein kinase (Bingham, 1976; Mackinlay *et al.*, 1977). Phosphorylated residues fulfilling such a requirement are present in α_{s1} -, β -, α_{s2} - and κ -caseins: all contain phosphoserine residues, the only known exception being one phosphothreonine residue in the very rare genetic variant D of α_{s1} -casein (Mercier *et al.*, 1971). Nevertheless, by using whole casein as a foreign substrate it is sometimes found that threonine residues are the major targets for some protein kinases, giving phosphothreonine/phosphoserine ratios higher than 1 (Takeda *et al.*, 1971; Matsumura & Takeda, 1972; Clari *et al.*, 1976).

The most obvious possible explanation for such a finding is that the site-specificity of the protein kinases phosphorylating threonine residues is different from that of the endogenous mammary-gland casein kinase. An alternative explanation, however, is suggested by the observation that several threonine residues that fulfil the requirement for attack by mammary-gland casein kinase but that are still not phosphorylated endogenously are available in the casein fractions: up to 6 in α_{s2} -casein (Brignon *et al.*, 1977), 2 in κ -casein (Mercier *et al.*, 1973) and 1 in both α_{s1} - and β -caseins (Mercier *et al.*, 1971; Ribadeau-Dumas *et al.*, 1972). It is possible therefore that protein kinases displaying a site-specificity similar to that of endogenous casein kinase are forced to phosphorylate such threonine residues unless serine residues have been made

available as well, e.g. by previous dephosphorylation.

In order to discriminate between these two possibilities we have studied in detail the phosphorylation of serine and threonine residues of native and partially dephosphorylated casein fractions by a rat liver cyclic AMP-independent protein kinase (so-called 'casein kinase' TS) (Meggio *et al.*, 1977), which is known to promote preferential phosphorylation of threonine residues in whole casein (Clari *et al.*, 1976). The results reported in the present paper clearly show that phosphorylation of threonine residues is abolished by previous enzymic dephosphorylation and support the conclusion that rat liver 'casein kinase' TS displays a site-specificity similar to that of mammary-gland casein kinase, preferentially phosphorylating serine residues of casein fractions whenever they are available.

Experimental

Rat liver protein kinase ('casein kinase' TS) was prepared from the soluble cytosol by combined Sepharose 6B and phosphocellulose chromatography, as previously described (Meggio *et al.*, 1977).

Whole casein was either 'Hammarsten casein' from Merck, Darmstadt, Germany, or it was prepared by acid precipitation at 30°C (McKenzie, 1967) from the skimmed milk of a single cow of Bavarian breed. α_{s1} - and α_{s2} -caseins were prepared from acid casein as described by Mercier *et al.* (1968) and Brignon *et al.* (1976) respectively. Samples of purified casein fractions were also kindly supplied by Dr. B. Ribadeau-Dumas (INRA, Jouy-en-Josas, France). All casein samples were also submitted to the same treatment as the partially dephosphorylated caseins (see below), omitting the addition of acid phosphatase.

Partial enzymic dephosphorylation of caseins was performed by incubation for various periods of time with potato acid phosphatase (Boehringer; grade II) as described by Brignon *et al.* (1977). Reactivity with ninhydrin did not increase during incubation, thus ruling out the occurrence of any appreciable proteolysis. The reaction was stopped by adding trichloroacetic acid to a final concentration of 5% (w/v), and the protein was recovered by centrifugation at 3000 rev./min for 10 min. The resulting pellet was dissolved in 0.5 M-Tris/HCl, pH 7.5, dialysed overnight against water and freeze-dried. The exact extent of dephosphorylation was determined by comparing the protein-bound phosphate, measured after HClO₄ digestion as described by Wagner (1960), in the original and dephosphorylated samples.

The enzymic phosphorylation of caseins and dephospho-caseins was performed by a 10 min incubation (unless indicated otherwise) in the presence of 'casein kinase' TS (2–4 µg/ml), 50 µM-[γ -³²P]ATP (sp. radioactivity 25 µCi/µmol) and 12 mM-MgCl₂ at pH 7.5, under the conditions previously described (Meggio *et al.*, 1978); casein concentration was 0.5 mg/ml unless indicated otherwise.

Serine-³²P ([³²P]phosphoserine) and threonine-³²P ([³²P]phosphothreonine) were isolated and quantified after 4 h hydrolysis in 6 M-HCl at 110°C of the labelled

caseins and pH 1.9 high-voltage paper electrophoresis [formic acid/acetic acid/water, (25:78:897, by vol.); 150 V/cm; 150 min] together with spots of authentic phosphoserine, phosphothreonine and P_i.

Only three radioactive peaks were constantly detected by a radiochromatoscanner, corresponding to P_i, phosphoserine and phosphothreonine; the radioactive areas corresponding to phosphoserine and phosphothreonine were cut out and counted for radioactivity in a liquid scintillator (Beckman LS-100C). The experimentally determined radioactivity was corrected for hydrolytic losses of 48 and 14% for serine-³²P and threonine-³²P respectively (Bylund & Huang, 1976).

Conditions for 7.5% (w/v) polyacrylamide-gel electrophoresis in 5 M-urea at pH 8.9 were those described previously (Donella Deana *et al.*, 1978), except that 0.5% mercaptoethanol was also present in both the gel and the buffer. In some runs 2 mM-MgCl₂ was added to minimize the contribution of phosphate groups to the electrophoretic mobility.

Results and Discussion

Rat liver 'casein kinase' TS produces in whole casein as well as in purified α_{s1} - and α_{s2} -caseins threonine-³²P/serine-³²P ratios higher than 1. As shown in Table 1, however, the previous partial dephosphorylation of the substrates promotes two parallel effects: (1) an almost complete prevention of

Table 1. 'Casein kinase' TS-dependent phosphorylation of threonine and serine residues of native and partially dephosphorylated casein fractions

General conditions are described in the Experimental section, which apply in all experiments, except for Expt. 2 in which [γ -³²P]ATP was 2 mM with a specific radioactivity of 1.5 µCi/µmol, and the addition of protein kinase was repeated after 2 and 4 h. Incubation time was 30 min unless indicated otherwise. The values in parentheses are molar ratios of ³²P/casein. The threonine-³²P/serine-³²P ratio was practically independent of the incubation time, between 10 min and 6 h.

Expt.	³² P incorporated (c.p.m./0.25 mg of casein)	Threonine- ³² P/serine- ³² P ratio
(1) Whole casein, native	73110 (0.125)	2.09
Whole casein, 50% dephosphorylated	182560 (0.313)	0.06
α_{s1} -Casein, native*	15440 (0.026)	1.97
α_{s1} -Casein, 50% dephosphorylated	107000 (0.183)	≤0.01
α_{s2} -Casein, native†	76800 (0.139)	1.05
α_{s2} -Casein, 30% dephosphorylated	240300 (0.436)	0.02
(2) α_{s1} -Casein, native, 1 h incubation	2320 (0.066)	1.90
α_{s1} -Casein, native, 2 h incubation	2275 (0.065)	1.87
α_{s1} -Casein, native, 6 h incubation	2345 (0.067)	1.95
α_{s1} -Casein, 50% dephosphorylated, 1 h	11017 (0.315)	≤0.01
α_{s1} -Casein, 50% dephosphorylated, 2 h	15837 (0.493)	≤0.01
α_{s1} -Casein, 50% dephosphorylated, 6 h	24897 (0.712)	≤0.01
(3) α_{s1} -Casein, native	16800 (0.028)	1.85
α_{s1} -Casein, 9% dephosphorylated	60520 (0.103)	0.19
α_{s1} -Casein, 13% dephosphorylated	71200 (0.122)	0.02

* Mol.wt. 23 600, containing 8 phosphate groups per molecule (Mercier *et al.*, 1971).

† Mol.wt. 25 000, containing 11–13 phosphate groups per molecule (Brignon *et al.*, 1977).

phosphorylation* of threonine residues and (2) a remarkable stimulation of overall phosphorylation, involving now almost exclusively serine residues.

Such a stimulation in turn is accounted for by two contaminant effects of the previous dephosphorylation: (a) an increased number of phosphorylatable sites, as evidenced by the prolonged incubation experiments of Table 1, and (b) a manifold enhancement of the phosphorylation rate, as can be deduced by the kinetic experiments illustrated in Fig. 1. It should be noted, however, that, although the $V_{\max}$ is greatly increased by the previous dephosphorylation, the K_m remains unchanged, suggesting that the binding of caseins to the protein kinase is not significantly affected by its degree of phosphorylation.

All the above results are fully consistent with the hypothesis that the serine residues phosphorylated in the native caseins, or at least some of them, become available to rat liver 'casein kinase' TS once they have been previously dephosphorylated. When they are in the native phosphorylated form, only threonine residues are available in large numbers, and therefore, whenever native casein is used as the substrate, preferential labelling of threonine residues is observed. Such a conclusion is also quite consistent with the finding that threonine residues susceptible to phosphorylation by our enzyme, i.e. threonine-130 of α_{s2} -casein (Meggio *et al.*, 1978) and threonine-49 of α_{s1} -casein (A. Pinna, A. Donella Deana & F. Meggio, unpublished work), are also both potential sites of endogenous phosphorylation, because of the presence of an acidic group two residues to their C-terminal side [see Brignon *et al.* (1977) and Mercier *et al.* (1971) respectively]. Moreover, the serine residues affected by our enzyme in both native and dephosphorylated α_{s1} - and α_{s2} -caseins belong to the typical [Ser(P)]₃ clusters that are also phosphorylated endogenously (A. Donella Deana, F. Meggio & L. A. Pinna, unpublished work). The results of Fig. 1 also show that α_{s2} -casein, which has a lower K_m , behaves as much more competitive substrate than α_{s1} -casein, thus accounting for the previously reported almost

Fig. 1. Kinetic behaviour of 'casein kinase' TS toward (a) α_{s1} - and (b) α_{s2} -casein

○, Native α_{s1} -casein; ●, 50%-dephosphorylated α_{s1} -casein; △, native α_{s2} -casein; ▲, 30%-dephosphorylated α_{s2} -casein. The total c.p.m. incorporated into the casein substrates are shown on the ordinate. In the insets, Lineweaver-Burk double-reciprocal plots are shown. By using the Eisenthal & Cornish-Bowden (1974) method essentially the same kinetic parameters were obtained.

negligible labelling of the latter whenever whole casein is submitted to enzymic phosphorylation (Donella Deana *et al.*, 1978; see also the footnote of the present paper).

The dramatic increases in the overall rates of phosphorylation caused by the previous dephosphorylation of phosphoserine residues also suggested that these might be more susceptible than threonine residues to phosphorylation by 'casein kinase' TS, thus slowing down, through a competitive mechanism, the labelling of the latter. Further strong support to such a conclusion is provided by the finding that even slight dephosphorylation of about 10%, expected to make available an average of 0.8 serine residue per molecule, is sufficient to lower the threonine- ^{32}P /serine- ^{32}P ratio of α_{s1} -casein from 1.8 to about 0.2 (see Table 1, Expt. 3). Assuming that only one threonine per molecule is available (threonine-49) and that the number of serine residues available to the enzyme before dephosphorylation was negligible compared with that made available by dephosphorylation, one can calculate that the rate of phosphorylation of serine residues by our protein kinase must be roughly five times higher than that of

* In the case of whole casein, the decrease in the threonine- ^{32}P /serine- ^{32}P ratio is also accompanied by improved phosphorylation of α_{s1} -casein (which is very poorly labelled in native whole casein), as evidenced by electrophoresis in the presence of 2 mM-MgCl₂ (to abolish the contribution of the phosphate groups to the mobility of the phosphoproteins) (Donella Deana *et al.*, 1978). Whenever α_{s1} - and α_{s2} -caseins are the substrates, however, the same band is found to be labelled before and after dephosphorylation, indicating that the threonine- ^{32}P and the serine- ^{32}P residues belong to the same substrate. A dephosphorylation-dependent decrease in the threonine- ^{32}P /serine- ^{32}P ratio is also found with β - and κ -casein preparations; in both instances, however, the radioactivity incorporated is largely accounted for by contamination (possibly due to α_{s2} -casein subfractions) and was not further investigated.

threonine residues, under conditions in which the concentrations of available serine and threonine residues are almost the same. This finding also indicates that caution should be exercised in the interpretation of the threonine- ^{32}P /serine- ^{32}P ratios produced by different protein kinases, since they may vary dramatically owing to small differences in the degree of phosphorylation of the casein preparations used. We have obtained, for instance, markedly lower ratios with commercial Hammarsten casein than with whole acid caseins carefully prepared from fresh milk in our laboratory or obtained from Dr. B. Ribadeau-Dumas.

In the light of the above results therefore we can conclude that there is no experimental support for any claim about different residue- or site-specificities between rat liver 'casein kinase' TS and the mammary-gland casein kinase(s), in spite of the fact that the former affects threonine residues of native casein fractions whereas the latter *in vivo* almost exclusively phosphorylates serine residues. Indeed once the suitable serine residues phosphorylated endogenously are made available by previous dephosphorylation, rat liver 'casein kinase' TS displays a remarkable preference for them and, moreover, the threonine residues phosphorylated in the absence of suitable serine residues still fulfil the structural requirements for the mammary-gland casein kinase(s).

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