Autophosphorylation of type 2 casein kinase TS at both its α- and β-subunits

Influence of different effectors

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Rat liver casein kinase TS (Ck-TS) having quaternary structure $\alpha_2\beta_2$, autophosphorylates at its 25 kDa, β-subunits, incorporating up to 1.2 mol P/mol enzyme. According to their effects on the autophosphorylation pattern the effectors of Ck-TS activity can be grouped into 3 classes: (i) inhibitors, like heparin, which also prevent the autophosphorylation of the β-subunit; (ii) stimulators possessing several amino groups (like spermine) which increase the autophosphorylation at the β-subunit; (iii) stimulators possessing several guanido groups, like protamines and related peptides, which prevent the phosphorylation of the β-subunit, while promoting the autophosphorylation of the 38 kDa α-subunit. In the presence of such polyarginyl effectors the 130 kDa Ck-TS is converted into forms with higher sedimentation coefficient.

Protein kinase *Casein kinase* *Protein phosphorylation* *Autophosphorylation* *Polyarginyl effector* *(Polyamine)*

1. INTRODUCTION

It is generally accepted that protein phosphorylation and dephosphorylation, catalyzed by protein kinases, represents one of the most widespread, general mechanisms of regulation (e.g., [1,2]). However, while in some instances the sequence of events responsible for the modulation of these enzymes has been elucidated (e.g., [3]), several protein kinases are known whose regulatory mechanisms, and hence physiological role, are still obscure.

A notable example is provided by the so-called casein kinases which are ubiquitous and apparently multi-substrate enzymes unaffected by cyclic nucleotides as well as by any other known second messengers (review [4]). In particular type-2 casein kinases there are oligomeric enzymes whose quaternary structure [4,5], site specificity [6] and some physiological substrates [7–10] are already known, whilst the mechanism of their regulation is not yet understood.

A common feature of type 2 casein kinases is the autophosphorylation of their small, β-subunit, which is devoid of catalytic activity and thus presumably plays a regulatory role [4,5]. On the other hand several compounds have been shown to affect in vitro this type of casein kinase, including heparin, a powerful and specific inhibitor [11–13], and several polycations which stimulate the enzymatic activity [14–16]. We have now investigated the possible correlation between these effectors and the mechanism of autophosphorylation of casein kinase-TS (Ck-TS), a type 2 casein kinase purified from rat liver cytosol.

This study shows that protamine, which stimulates Ck-TS activity, also promotes a remarkable autophosphorylation of the catalytic α-subunits of the enzyme, while depressing the phosphorylation of the β-subunit. Structural

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modifications are also induced resulting in polymeric form(s) with higher sedimentation coefficients. The effect of protamine can be mimicked by other polyarginyl peptides, but neither by free arginine nor by polyamines like spermine.

2. MATERIALS AND METHODS

Type 2 casein kinase TS (Ck-TS) was purified nearly to homogeneity from rat liver cytosol by phosphocellulose chromatography followed by Sepharose 6B gel filtration, as in [8]. The enzyme used in most autophosphorylation experiments was also subjected to sucrose gradient ultracentrifugation in order to remove a couple of minor phosphorylatable contaminants.

Casein kinase activity was tested routinely using whole casein as phosphorylatable substrate, as in [8]; $MgCl_2$ was 2 mM in those experiments where spermine was also present, to optimize the stimulation by this effector [16].

Autophosphorylation of Ck-TS was performed by incubating the enzyme (20–40 ng) at 37°C for 15 min (unless differently indicated) in 200 μl of a medium containing: 100 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM NaCl and 5 μM [γ-^{32}P]ATP with a specific radioactivity of about 2 μCi/nmol. In some experiments NaCl was omitted and $MgCl_2$ was 2 mM. The concentration of effectors, whenever present, is specified in the figures. The incubation was stopped with trichloroacetic acid (final conc. 10%); 100 μg phosvitin were added as carrier and the radiolabeled Ck-TS was recovered by centrifugation. After 3 washings with trichloroacetic acid the pellet was dissolved with the minimum volume of 0.1 M NH_4HCO_3. Comparable aliquots were subjected to electrophoresis on vertical plates of 10% polyacrylamide containing SDS, prepared essentially as in [17], following [8]. The slabs were stained either with Coomassie blue or silver stain (Biorad), dried and autoradiographed to evidence the ^{32}P incorporated into the subunits of Ck-TS. Alternatively, in order to obtain a still active autophosphorylated holoenzyme, at the end of incubation (performed at 2°C) the radioactive sample was applied to a 60 × 1.8 cm Sephadex G25 column equilibrated and operated at 2°C with 0.1 M Tris–HCl (pH 7.5), 0.5 M NaCl, 50 μM PMSF and 0.01% Brij 35. The peak of autophosphorylated Ck-TS, eluted at the void volume, was collected and utilized for further characterizations.

The isolation of radiolabeled subunits was performed by subjecting autophosphorylated Ck-TS to gel electrophoresis on a 7 × 1 cm column of 10% polyacrylamide in the presence of 0.1% SDS, prepared and operated essentially as the plates. The column was cut into 2-mm slices which were submitted to Cerenkov counting. The slices corresponding to the autophosphorylated subunits were eluted as in [18].

^{32}P-peptide mappings of the radiolabeled subunits were performed by 15% polyacrylamide gel electrophoresis after limited digestion with *Staphylococcus aureus* V8 protease, essentially as in [19].

Sucrose gradient (5–20%) ultracentrifugation took place in a Beckman SW 40 rotor, for 18 h as in [20].

3. RESULTS

3.1. *Quaternary structure of Ck-TS and phosphorylation of its β-subunit*

As shown in fig.1 the M_r of Ck-TS as determined by sucrose gradient ultracentrifugation in 0.4 M KCl is slightly lower than that of the marker protein lactate dehydrogenase (140000) thus fitting quite well with the value of 130000 calculated by gel filtration [8].

Polyacrylamide gel electrophoresis in SDS of the enzyme recovered from sucrose gradient ultracentrifugation shows two bands of M_r 38000 and 25000 (fig.1B, inset). In some preparations however, an additional 36000 band is also detectable. Silver staining reveals the 25000 band much better than Coomassie blue. Densitometric analyses of silver-stained gels indicate a ratio of 25 kDa to 38 kDa of 0.9–1.2. The 25 kDa band becomes labeled upon incubation of purified Ck-TS with [γ-^{32}P]ATP in a similar manner to other type II casein kinases. Autophosphorylation occurs also at low temperatures: in particular after 2–6 h incubation at 3°C with [γ-^{32}P]ATP and Mg^{2+}, it is possible to obtain fully active preparations of Ck-TS which have incorporated 0.7–1.2 mol ^{32}P/mol enzyme. The ^{32}P-radioactivity comigrates with the casein kinase activity on sucrose gradient ultracentrifugation (fig.1B) and Sephacryl S-200 gel filtration (not

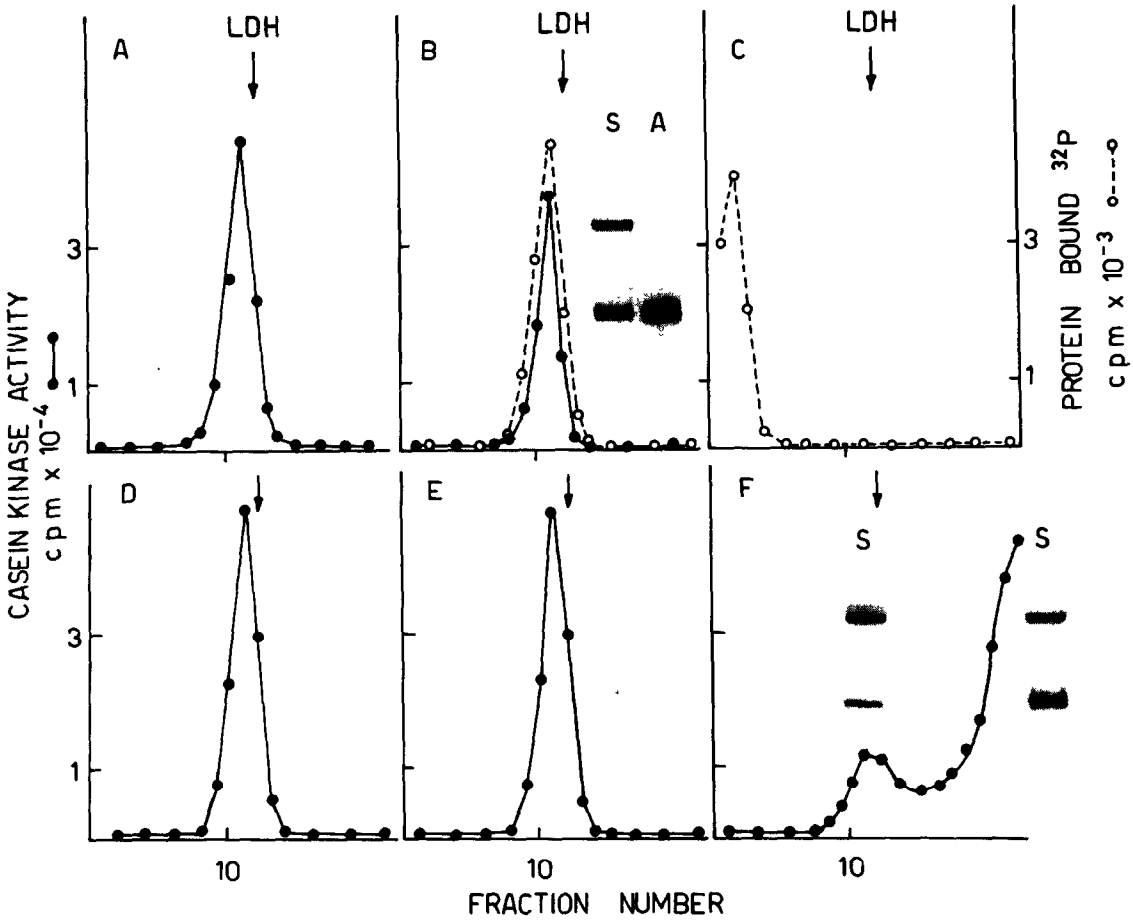

Fig.1. Sucrose gradient ultracentrifugation of Ck-TS and its β-subunit, under different conditions: (——) casein kinase activity; (···) protein-bound ^{32}P; (——→) position of lactate dehydrogenase, M_r 140000. (A) Unlabeled Ck-TS; (B) [^{32}P]Ck-TS, autophosphorylated at its β-subunit at 3°C for 8 h; (C) ^{32}P-labeled β-subunit prepared as in B and isolated by preparative polyacrylamide gel electrophoresis in SDS (see section 2); (D) Ck-TS preincubated 5 min with heparin (1 μg/ml); (E) Ck-TS preincubated 5 min with spermine (2 mM); (F) Ck-TS preincubated 5 min with protamine (0.2 mM). The insets refer to SDS–PAGE patterns of Ck-TS fractions after ultracentrifugation: S, silver staining; A, autoradiography.

shown) rather than moving in the position of the isolated 25 kDa component.

If the 38 kDa and 25 kDa bands are designated as α and β, respectively, the most likely quaternary structure of rat liver Ck-TS is $\alpha_2\beta_2$. The resulting M_r of the holoenzyme would be 125000 which is in very good agreement with the experimental value of 130000 found both by ultracentrifugation and gel filtration. As also shown in fig.1 the ultracentrifugation pattern of Ck-TS is not altered by preincubation with inhibitors like heparin and polyamine stimulators like spermine. It is strongly affected however by preincubation with protamine which gives rise to a peak of activity close to the bottom of the tube. Such heavy form(s) of Ck-TS still include both α- and β-subunits in the same ratio as the 'native' 130 kDa Ck-TS. Moreover after a second sucrose gradient ultracentrifugation, 'heavy' Ck-TS was largely converted back to the 130 kDa form (not shown).

3.2. *Influence of different effectors on Ck-TS autophosphorylation*

The influence of various effectors on Ck-TS autophosphorylation is shown in fig.2. Heparin and polyglutamic acid, both specific and powerful inhibitors of Ck-TS activity ([13]; unpublished data), prevent the radiolabeling of the β-subunit by [γ-^{32}P]ATP. This has also been found with unlabeled GTP which serves as phosphate donor for type 2 casein kinases. Spermine, which stimulates the casein kinase activity of Ck-TS in vitro causes a slight increase in the autophosphorylation of the β-subunit. In contrast, the effect of protamine, which also stimulates casein kinase activity in vitro, is unusual in that it reduces the phosphorylation of the β-subunit while promoting the phosphorylation of one or two radiolabeled bands of M_r 38000 (and 36000) corresponding to the α-subunit(s) of Ck-TS. A similar effect is seen on addition of synthetic nona- to tetradeca-peptides representing fragments of the protamine galline, which contains several consecutive arginine residues. Neither polylysine, free arginine, nor the pentapeptide Ser–Gly–Arg–Arg–Arg, can induce the same shift on the autophosphorylation pattern, at comparable concentrations of basic residues (fig.2).

A close parallel is observed between stimulation of enzymatic activity and effect on autophosphorylation induced by increasing concentrations of protamine (fig.3). It should be stressed on the other hand that the disappearance of the 25 kDa radiolabeled band promoted by poly(arginyl) effectors is not paralleled by any decrease in the amount of the β-subunit as judged by its intensity after either Coomassie blue or silver staining (fig.2A,B) thus providing the unambiguous demonstration that these effectors actually prevent the phosphorylation of the β-subunit rather than either promoting its disappearance or altering its electrophoretic mobility.

Moreover, as shown in fig.4, ^{32}P-peptide maps obtained after *Staphylococcus aureus* protease digestion of either the α- or β-subunits radiolabeled in the absence and presence of protamine, respectively, are markedly different, thus ruling out any similarity between the phosphorylation sites of these subunits.

The radiolabeling of both the α- and β-subunits resembles in several respects the phosphorylation

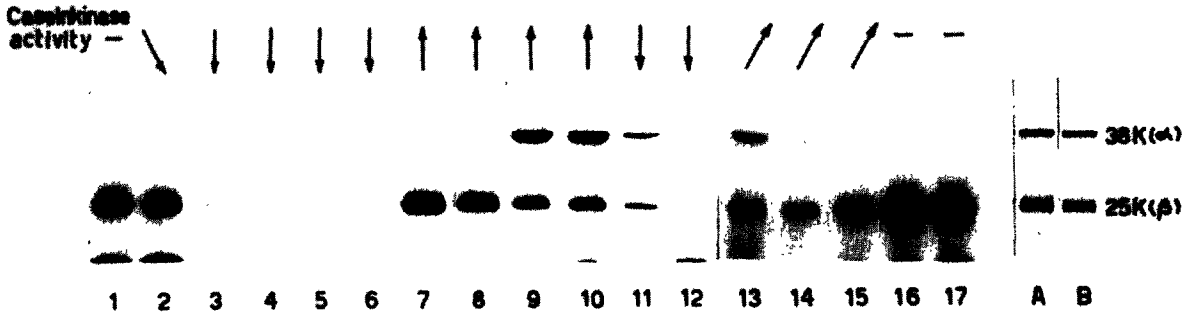

Fig.2. Autophosphorylation of Ck-TS at its β- and α-subunits. Influence of different effectors. General conditions for autophosphorylation and gel electrophoresis of radiolabeled Ck-TS are those in section 2 except for the omission of NaCl from the incubation medium. Numbers denote autoradiographies while letters A and B denote the silver staining corresponding to the autoradiographies 1 and 9, respectively. Downward and upward arrows denote inhibition and stimulation respectively of casein kinase activity under the experimental conditions used for autophosphorylation: (1) control; (2) control + 2 mM $MgCl_2$; (3) – $MgCl_2$; (4) + heparin (1 μg/ml); (5) + polyglutamate (20 μg/ml); (6) + unlabeled GTP (0.5 mM); (7) + spermine (2 mM) with 2 mM $MgCl_2$; (8) + polylysine (0.2 mg/ml); (9) + protamine (salmine, 30 μM); (10) 5 min incubation with unlabeled ATP (1 mM) and ADP (5 mM) after incubation with protamine and [γ-^{32}P]ATP as in 9; (11) + protamine (30 μM) + GTP (0.5 mM); (12) + protamine (30 μM), – $MgCl_2$; (13) + peptide: $(Arg)_4$–Tyr–Gly–Ser–$(Arg)_6$–Tyr (0.2 mM); (14) + peptide $(Arg)_4$–Ala–Gly–$(Arg)_4$ (0.2 mM); (15) + peptide Gly–Ser–$(Arg)_6$–Tyr (0.5 mM); (16) + peptide Gly–Ser–$(Arg)_3$ (0.8 mM); (17) + arginine (1.5 mM).

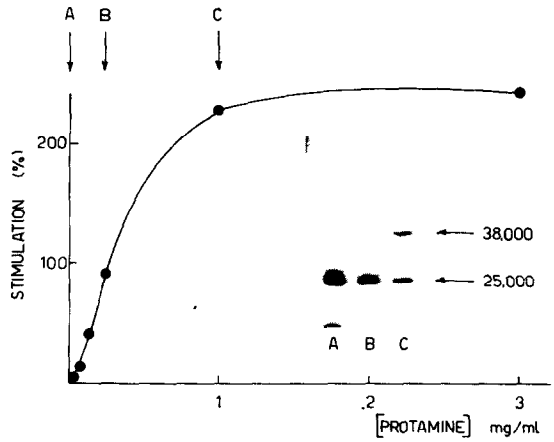

Fig.3. Stimulation of casein kinase activity and inhibition of β-subunit autophosphorylation by increasing concentrations of protamine. The inset shows the autoradiographies of Ck-TS autophosphorylated either in the absence of protamine or in the presence of the indicated concentrations of protamine, and subjected to SDS–PAGE.

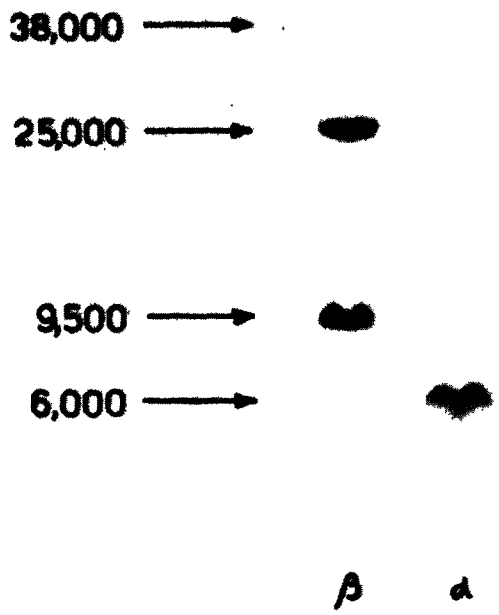

Fig.4. ^{32}P-peptide maps of autophosphorylated β- and α-subunits of Ck-TS after limited proteolysis with *Staphylococcus aureus* protease. The α- and β-subunits were phosphorylated in the absence and presence of 30 μM protamine, respectively. Conditions for the isolation of radiolabeled subunits and peptide mapping are detailed in section 2.

of extrinsic protein substrates, like casein: in particular it requires Mg^{2+}, it is prevented by excess unlabeled GTP and it involves Ser-P residue(s) (not shown) unable to exchange with unlabeled ATP or to transfer their phosphate to ADP.

4. DISCUSSION

To our knowledge this is the first report describing the autophosphorylation of a type-2 casein kinase at its α-subunits in response to stimulators of enzymatic activity promoting both a concomitant decrease of the β-subunit phosphorylation and a drastic modification in the structure of the holoenzyme.

In the absence of any effectors, rat liver casein kinase TS (Ck-TS) exhibits its usual M_r of 130000 and undergoes autophosphorylation almost exclusively at its small (25 kDa) subunit. Even when fully phosphorylated, such a 25 kDa subunit comigrates with the 130 kDa holoenzyme, thus confirming unambiguously that it actually participates in its quaternary structure rather than representing a tightly bound phosphorylatable substrate.

In addition, many effectors of Ck-TS activity influence its autophosphorylation pattern. Depending on their different effects and chemical properties they can be grouped into 3 classes:

(i) Inhibitors, such as heparin and polyglutamic acid which also prevent the β-subunit autophosphorylation without eliciting any autophosphorylation of the α-subunit;

(ii) Polycations with amino groups, such as spermine and polylysine which stimulate the casein kinase activity and slightly increase the β-subunit autophosphorylation as well;

(iii) Polycations with guanido groups, like protamines and other arginine-rich peptides which also stimulate the casein kinase activity but actually prevent the β-subunit autophosphorylation while promoting a concomitant autophosphorylation of the α-subunit.

Such effectors also specifically convert the 130 kDa Ck-TS into a larger form(s) with higher sedimentation coefficient. All these effects cannot be mimicked by either free arginine or the pentapeptide Gly–Ser–$(Arg)_3$. This would indicate that a relatively large number of structurally

organized guanidinyl groups is required for promoting them.

Although the actual physiological relevance of the above described effects is not yet proved, it should be mentioned that a faint but significant autophosphorylation of the α-subunit could be detected in several preparations of Ck-TS even without addition of polyarginyl effectors, while a type-2 casein kinase isolated from yeast has been reported to undergo spontaneously a remarkable phosphorylation at a 35 kDa polypeptide probably corresponding to its large subunit [23]. It is conceivable therefore that the α-subunit autophosphorylation might have functional significance.

On the other hand the possibility that α-phosphorylated Ck-TS might be an intermediate of the catalytic reaction is contradicted by the stability of its phosphorylated residue(s), which is/are unable both to exchange with ATP and to transfer their phosphate to ADP (see fig.2, lane 10).

It has been recently shown that the β-subunit of type 2 casein kinase from lung, albeit devoid of intrinsic catalytic activity, is required in order to optimize the catalytic activity of the α-subunits [5]. It is conceivable therefore that protamine and related peptides may favour a more suitable association of the β- with the α-subunits, thus improving the catalytic efficiency. The altered sedimentation coefficient and autophosphorylation pattern induced by polyarginyl effectors are quite consistent with such a hypothesis, although it is not yet clear whether the structural modifications and the stimulatory effect are causally related.

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