RESEARCH COMMUNICATION

A polylysine-induced aggregation of substrate accompanies the stimulation of casein kinase II by polylysine

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Casein kinase II (CK-II) activation by polylysine parallels an aggregation of substrates promoted by the polycation. CK-II is known to be stimulated by basic polypeptides and polyamines. The mechanism by which this stimulation takes place, however, is not yet fully understood. Here we show that, in the usual CK-II assay, polylysine induces the aggregation of casein. This aggregation has been monitored by turbidimetry, electron microscopy and gel filtration. The polylysine-concentration-dependence of the casein aggregation parallels the polylysine-

concentration-dependence of the enzyme stimulation. In the presence of polylysine the enzyme is incorporated into the casein aggregates promoted by the polycation, thus supporting the view that this substrate aggregation is directly related to the mechanism of CK-II stimulation. Preliminary results show that a similar parallelism occurs with other natural substrates of the enzyme. The physiological meaning of this substrate aggregation, and its possible relation to other polylysine-stimulated enzymes and polylysine-aggregated proteins, are discussed.

INTRODUCTION

Casein kinase II (CK-II) is an ubiquitous protein kinase independent of cyclic nucleotides and Ca^{2+} ions, most probably involved in the control of many metabolic events and pathways, in the transduction of extracellular signals, as well as in those processes related to cell differentiation and proliferation (for reviews, see [1–4]). A number of substances have been long known to affect the CK-II activity *in vitro*. Many of the inhibitors are polyanions, and basic polypeptides and polyamines are stimulators [5–7]. In spite of the extensive accumulation of data, the information concerning the mechanism of the stimulatory action is largely phenomenological, and the extent of stimulation depends on various factors [3,4,8].

Partially dephosphorylated whole casein is one of the most commonly used proteins in routine assays of CK-II. This protein substrate is a heterogeneous group of phosphoproteins displaying strong interacting effects leading to aggregates of variable size, depending on the experimental conditions [9–11]. A strong aggregation of casein was repeatedly observed in our laboratory to be promoted by polylysine, at concentrations currently used to activate CK-II. This observation led us to investigate the possibility that the mechanism of polylysine stimulation of CK-II is mediated by a polylysine-induced aggregation of substrates. Herein we present the results of polylysine stimulation of CK-II activity concomitant with a casein aggregation promoted by the polycation. Preliminary results obtained with physiological substrates of the enzyme are also included.

MATERIALS AND METHODS

Materials

Polylysine (P-4408), Sepharose 6B, heparin–acrylic, hydroxyapatite and Tris were from Sigma. DEAE-cellulose and phosphocellulose were from Whatman. ATP was purchased

from Boehringer-Mannheim, and NaCl and MgCl_2 were from Merck. Radiolabelled ATP was from Amersham International. Casein (Sigma C-5890) was partially dephosphorylated by using the procedure described by Reimann et al. [12]. The microtubule-associated proteins (MAPs) were prepared as described by Vallee [13]. Taxol was generously provided by Nancita R. Lomax of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment. The regulatory subunit of the cAMP-dependent protein kinase (R) was purified according to Beavo et al. [14] and Rannels et al. [15].

Enzyme preparation

Bovine brain CK-II was partially purified by using essentially the method of Alcázar et al. [16] with some modifications. Hydroxyapatite and heparin–acrylic were used instead of ATP–agarose and phosvitin–Sephacrose. The enzyme was further characterized by immunoblotting as described by Towbin et al. [17]. The first antibody was generously given by Professor Dahmus of the University of California, Davis, CA, U.S.A. The enzyme was routinely assayed at 37 °C in a final volume of 50 μl containing 20 mM Tris, 8 mM MgCl_2 , 2 mM EGTA, 0.16 M NaCl and 20 μM ATP (1 μCi), pH 7.2, unless otherwise indicated. The assays were initiated by adding ATP and stopped by precipitation with 10% trichloroacetic acid. The specific activity was about 350 nmol of phosphate incorporated into casein/min per mg of enzyme, in the absence of stimulator. SDS/PAGE was performed by the Laemmli [18] procedure. In some cases the gels were dried, cut into segments and Čerenkov-counted. Protein concentrations were measured by the methods of Bradford [19] and Lowry et al. [20], using BSA and casein as references.

Turbidimetry

Turbidity measurements were carried out at 300 or 350 nm in a Perkin–Elmer spectrophotometer equipped with a thermostatic

cell holder, at 37 °C. Turbidity measurements and activity assays were initiated after 15 min of sample preincubation at 37 °C.

Electron microscopy

Samples were prepared by placing a drop of the corresponding solution on to a carbon–collodion electron-microscopy grid. The protein was then shadowed with uranyl acetate and observed in a JEOL 100B electron microscope.

Gel filtration

Protein solutions were fractionated on a column (600 mm × 12 mm) of Sepharose 6B at room temperature.

RESULTS

Figure 1 shows the effect of polylysine on CK-II activity at increasing casein concentrations. The turbidity generated by casein is also included. The enzyme activity showed a behaviour clearly sigmoidal with respect to casein, with Hill coefficients (h) of 1.7 and 2.2 in the absence or presence of 0.03 mg/ml polylysine respectively. These values suggest a co-operative interaction of the enzyme with its protein substrate, although an h value higher than 2 would be incompatible with a two-catalytic-subunit holoenzyme. In the absence of polylysine, increasing concentrations of casein produce a small level of turbidity, probably due to the well-known tendency of casein molecules to associate. In the presence of polylysine the turbidity becomes much greater and complex. At casein concentrations below about 0.1 mg/ml, where the CK-II activity is low, a considerable turbidity develops. On increasing the casein concentration the turbidity declines, and above about 0.5 mg/ml a new aggregation process seems to take place.

Electron microscopy indeed bore out that at least two types of casein aggregation are promoted by polylysine, depending on

the casein concentration. When the latter is low, polylysine induces casein to aggregate into very large rounded protein particles, which were observed under the microscope either isolated (Figure 2a) or in groups composed of a small number of them (Figure 2b). At a higher casein concentration, where polylysine clearly stimulates the enzyme, a completely different structure of the aggregates could be observed. Casein seems to be organized into smaller rounded particles, which in turn associate with each other to form either rosary-like structures composed of a large number of units (Figure 2d) or large massive associations (Figure 2e). Parts (c) and (f) of the same Figure show casein at low and high concentration, but in the absence of polycation.

In spite of the complex evolution of casein turbidity shown in Figure 1, some parallelism can be observed between turbidity and activity above 0.4 mg/ml casein. Figure 3 shows the turbidity of casein at 1 mg/ml and the activity of CK-II as a function of the polylysine concentration. As can be seen, the stimulation of the enzyme activity now parallels the development of turbidity promoted by the polycation. This parallelism could be observed also in an experiment in which enzyme activity and casein turbidity were measured in the presence of polylysine at increasing concentrations of heparin (Figure 4). When the concentration of this known inhibitor of the enzyme [5] was high enough to neutralize polylysine, both activity and turbidity fell. These results indicate that, at high casein concentration, the CK-II stimulation by polylysine occurs concomitantly with the development of a turbidity, which would be a consequence of the particular type of aggregation shown in parts (d) and (e) of Figure 2.

At low casein concentrations, where the polylysine stimulation hardly occurs at all, electron microscopy indicated a completely different type of aggregation, probably due to the high polylysine/casein ratio. The different subunits composing casein have a strong tendency to associate with one another to form highly organized structures. The size and structural organization of these aggregates depend on casein concentration, ionic strength, temperature and other experimental conditions [10,11]. It seems that, at low casein concentration, polylysine induces the formation of the large aggregates shown in parts (a) and (b) of Figure 2, which would account for the high increase in turbidity registered in Figure 1. These aggregates, however, would not have any relation to the enzyme stimulation. Only when the casein concentration is high enough does a different type of aggregation develop, and a parallelism between turbidity and polylysine stimulation can be observed.

The co-operative nature of the casein turbidity developed at increasing polylysine concentrations is noteworthy. In fact a Hill analysis of the turbidity data in Figure 3 gave a Hill coefficient (h) of 1.5. A similar co-operative nature could be observed whenever casein was induced to aggregate by different agents, such as Ca^{2+} or Mg^{2+} ions (results not shown). If not fortuitous, this co-operative nature of casein aggregation might account for the sigmoidal behaviour of the enzyme activity with respect to casein concentration.

CK-II has been reported to be associated into organized cell structures such as the mitotic spindle [21,22] and synaptosomes [23]. Also it co-polymerizes with clathrin-coated vesicles [24] and with microtubules obtained *in vitro* by taxol and GTP polymerization of tubulin [13]. The enzyme so associated phosphorylates proteins which are components of these organized structures. The possibility that CK-II becomes stimulated upon incorporation into the casein aggregates was investigated by gel filtration. A solution of CK-II and casein at 1.2 mg/ml was loaded on to a Sepharose 6B column. As can be seen in the elution pattern of Figure 5(b), essentially all the casein emerged

Figure 1 Effect of polylysine on the casein-concentration-dependence of the CK-II activity and casein turbidity

The enzyme was assayed in the absence (□) or in the presence of 0.03 mg/ml polylysine (○). The assays were stopped by adding 10% trichloroacetic acid. The samples were washed twice with the same acid solution, dissolved in Laemmli sample buffer [18], and counted for radioactivity. The specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was 750 c.p.m./pmol. The insets show the corresponding autoradiographies. Turbidity measurements were carried out on casein solutions prepared under conditions identical with those of the activity assays, except for the enzyme, which was omitted, in the absence (■) or in the presence of 0.03 mg/ml polylysine (●).

Figure 2 Electron micrographs of casein aggregates

Casein was prepared in the phosphorylation buffer at 0.1 mg/ml (**a**, **b** and **c**) or 1 mg/ml (**d**, **e** and **f**) in the absence (**c** and **f**) or in the presence (**a**, **b**, **d** and **e**) of 0.03 mg/ml polylysine. Parts (**a**) and (**b**), as well as (**d**) and (**e**), are two views of the same field. The bar represents 500 nm.

Figure 3 Polylysine-dependence of CK-II activity and of turbidity of casein

The enzyme was assayed in the presence of the indicated polylysine concentrations, using casein at 1.0 mg/ml and 0.11 M NaCl (○). The percentage of radioactivity was calculated with respect to the maximum of 3200 c.p.m. in the 30 kDa band of casein. The basal radioactivity in the absence of polylysine was 990 c.p.m. The inset shows the autoradiographic analysis. Turbidity measurements (●) are from 1 ml casein solutions (1.0 mg/ml) in the same phosphorylation buffer.

at a position consistent with the molecular mass described for the casein submicelles (300 kDa [10]) as deduced from a parallel run in which catalase was used as marker. A small fraction of about 7% of casein emerged at the exclusion limit of the column, indicating that, even in the absence of polylysine, some casein may self-associate into large aggregates. Aliquots of the eluted fractions were then assayed for CK-II activity, by adding an excess of fresh casein, polylysine and the usual assay medium. The elution pattern of the enzyme was essentially similar to that of casein. A small fraction of the activity was found at the exclusion limit, although most of the enzyme was found slightly earlier than casein. Figure 5(a) shows the same chromatographic

Figure 4 Heparin-dependence of CK-II activity and of turbidity of casein in the presence of polylysine

The enzyme was assayed using casein at 1 mg/ml in the presence of 0.03 mg/ml polylysine. Radioactivity values were obtained as described in Figure 1 and are expressed as the percentage of the value at the lower heparin concentration (○). Turbidity measurements (●) are from solutions of casein at the same concentration and in the same buffer.

Figure 5 Sepharose 6B filtration of casein aggregates

(a) Casein (1.2 mg/ml) in the phosphorylation buffer, was incubated for 15 min at 37 °C in the presence of polylysine at 0.05 mg/ml and CK-II at 1 μg/ml. After that time 0.5 ml of this solution was loaded on to the column. Fractions (1 ml) were collected and aliquots of them assayed for CK-II activity and protein concentration. (b) The experiment carried out under the same conditions, except for polylysine, which was absent. The percentages of activity were calculated with respect to that found in the fraction having the maximum value. ○, Enzyme activity; ●, casein/fraction; □, elution pattern of CK-II in the absence of casein, either with polylysine (**a**) or without polylysine (**b**). The arrows show the position at which catalase was eluted.

profile of casein and CK-II, but in the presence of polylysine at 0.05 mg/ml. In this case a higher proportion of casein (20%) was found in the exclusion limit. The rest of casein emerged with a strongly asymmetric peak centred at the same position found in (b). Most noteworthy was that essentially all the enzyme activity was found in those fractions emerging at the front, indicating that the high aggregates of casein incorporate most of CK-II. When CK-II was run in the absence of casein, a strong enzyme inactivation was observed in the elution fractions. The enzyme was eluted slightly earlier than catalase, but practically no activity was found in the exclusion limit of the column, irrespective of whether polylysine was absent or present.

Physiological substrates of CK-II (MAP 1A and R) displayed a behaviour similar to that of casein. Figure 6(a) shows the CK-

Figure 6 Turbidity of MAPs and phosphorylation by CK-II

(a) Turbidity was measured on MAP solutions at 0.4 mg/ml in the presence of the indicated polylysine concentrations (●). The CK-II activity associated with the MAP preparation was used as source of enzyme (○). The percentage of radioactivity was calculated with respect to the maximum of 4550 c.p.m. in the MAP 1A band. The basal radioactivity in the absence of polylysine was 1210 c.p.m. (b) Turbidity was measured on MAP preparations in the absence (■) and in the presence (●) of 0.02 mg/ml polylysine. Activity in the absence (□) and in the presence of 0.02 mg/ml polylysine (○) is expressed as the percentage of radioactivity with respect to the maximum of 2080 c.p.m. in the MAP 1A band.

Figure 7 Polylysine-dependence of the CK-II activity and of turbidity of MAP R

Turbidity (●) was measured on 0.1 mg/ml MAP R in the phosphorylation buffer containing 0.11 M NaCl at the polylysine concentrations indicated. CK-II was assayed under the same conditions (○). Radioactivity values were obtained as described in Figure 1, and are expressed as percentages with respect to the maximum value.

II activity on MAP 1A and the turbidity of the protein substrate solutions as a function of the polylysine concentration. Part (b) of the same Figure shows the same parameters at a fixed polylysine concentration, but as a function of the substrate concentration. In both cases a close parallelism could be observed between enzyme activity and the turbidity developed by the substrate. In the case of MAP R the extent of polylysine stimulation decreased considerably upon increasing the ionic strength. Figure 7 shows the CK-II activity on R and the turbidity measurements, after subtracting the corresponding values in the absence of polylysine. Again a parallelism could be observed between the extent of polylysine stimulation of the activity and the aggregation of the substrate.

DISCUSSION

Casein has a strong tendency to become aggregated, and it is not the natural substrate of CK-II. The facts, however, that (a) the polylysine stimulation of the enzyme parallels the formation of a particular type of casein aggregates, (b) the enzyme is incor-

porated into these aggregates and (c) the same parallelism between activity and turbidity is observed with physiological substrates of the enzyme strongly suggest that the mechanism of polylysine stimulation of CK-II is related to processes of substrate aggregation.

Polylysine has an intriguing ability to promote aggregation of a number of proteins of very different and apparently unrelated functions. Thus polylysine induces the aggregation of vesicles made of biological membranes or of phospholipid/protein mixtures [25]. Also, some membrane proteins of erythrocytes [26] undergo polylysine-induced aggregation. Polylysine has been described as aggregating the insulin receptor, and specifically to activate the insulin-dependent insulin receptor protein kinase [27,28]. Mellgren [29] has recently reported that polylysine activates a multicatalytic proteinase. The fact that casein is used as substrate of this enzyme would support the notion that the stimulatory effect of polylysine is mainly based on the casein aggregation promoted by the stimulator.

Tyrosine kinases [30], inositol phospholipid kinases [31] and phosphatases [32] have also been described as being susceptible to activation by polycations. The physiological meaning of this ability is far from being understood. It has been suggested that polycations mimic *in vitro* the action of some native components on CK-II *in vivo*. An alternative, although still highly speculative, suggestion is that, by means of the substrate aggregation, polylysine builds *in vitro* a particular structure able to simulate that in which the enzyme works *in vivo*. The tendency of CK-II to associate into organized supramolecular structures [13,21–24] supports the notion that the stimulatory action of polylysine may be based on the capacity of the polycation to aggregate the substrates into the organized structures in which the enzyme is then incorporated, thereby acquiring a more active conformation.

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