A surface-plasmon-resonance analysis of polylysine interactions with a peptide substrate of protein kinase CK2 and with the enzyme

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The mechanism of protein kinase CK2 (CK2) activity stimulation by polylysine has been studied by surface plasmon resonance (SPR). The kinetics of the polylysine interaction with a peptide substrate of the enzyme, and with the enzyme itself, have been investigated. A peptide containing a threonine (T) residue surrounded by a cluster of negatively charged acidic [arginine (R) and glutamic acid (E)] residues, RRREEETEEE, and specifically phosphorylated by CK2, was selected. Polylysine interacts with both the enzyme and the peptide substrate. The rate constant, the stoichiometry of the polylysine–peptide substrate interaction and

the kinetic parameters of the stimulated enzyme were used to calculate the polylysine-dependent stimulation of CK2. The results are in agreement with experimentally determined polylysine-dependent stimulation. The polylysine–enzyme interaction is too slow to account for enzyme stimulation. The behaviour of polylysine is not reproduced by the polyamine spermine. The results are consistent with a substrate-mediated mechanism of CK2 stimulation by polylysine, and they suggest that the CK2 stimulation by polyamines occurs by a different mechanism.

INTRODUCTION

Protein kinase CK2 (CK2) is a ubiquitous serine/threonine protein kinase. It catalyses the phosphorylation of a large number of substrates which may be involved in gene expression, cell proliferation and signal transduction (for reviews, see [1–5]). The enzyme has a heterotetrameric $\alpha_2\beta_2$ or $\alpha\alpha'\beta_2$ structure [6,7], with the catalytic activity residing in the α -subunits [8,9]. CK2 does not seem to be responsive to second-messenger regulators, and its physiological regulation remains puzzling and controversial.

The ability displayed by basic polypeptides like polylysine, and basic proteins like protamine, to stimulate enzyme activity *in vitro*, is one of the regulatory aspects that has deserved special attention during the last few years. The physiological significance of this stimulation, however, is far from being clear. Nevertheless, the existence of natural lysine-rich sequences in proteins such as histones and K-ras [10] protein suggests a plausible physiological up-regulation that would be mimicked *in vitro* by polycations like polylysine. Neither the mechanism through which the stimulation occurs *in vitro*, nor the primary event causing stimulation, are fully understood. It might be an interaction between the enzyme and the polycation, giving rise to an activated enzyme, but it could also be a substrate–polycation interaction, which would improve the susceptibility of the substrate to be phosphorylated. Even an interaction of stimulator with both enzyme and substrate cannot be ruled out [11]. Many reports supply data which point to a direct interaction of positively charged compounds with the β -subunit of the enzyme, producing an activated form of the α -subunit [12–15]. On the other hand, the idea that CK2 stimulation might be mediated by polycation–substrate interactions is also widely supported. For example, it has been reported that the polycation concentration needed to achieve 50% stimulation is substrate-dependent [16–18]. Also, the extent of stimulation has been shown to be dependent on the substrate used [16,18,19].

We have reported that substrate aggregation induced by

polylysine parallels the polylysine stimulation of CK2 activity [20]. The non-physiological substrate casein, and physiological substrates such as the regulatory subunit of cAMP-dependent protein kinase (pKA) and microtubule-associated protein 1 (MAP1), produce aggregation at the same polylysine concentrations at which activity stimulation is observed. These results support the notion that the polycation stimulation of CK2 may be substrate-mediated. Polylysine stimulation, however, is very strong when low-molecular-mass substrates are used [18]. This is the case with the RRREEETEEE peptide. It is a specific substrate of the enzyme [21] and it contains a threonine (T) residue surrounded by a cluster of acidic residues (R, arginine; E, glutamic acid). Maximal stimulation is obtained at a very low molar concentration of polylysine. Using peptide substrates at 1 mM, activity stimulation by polylysine is reported even in the micromolar range of polycation concentration [18]. These observations do not seem to be reconcilable with a model of CK2 stimulation based on a polylysine–substrate complex. In the present study we have used surface plasmon resonance (SPR) in order to investigate the possibility that polylysine may interact with the peptide substrate RRREEETEEE. The results show that such interaction occurs. The nature of the polylysine–peptide interaction and its relation to the enzyme stimulation are also presented. As far as we know, there are no physico-chemical data concerning polylysine interaction with CK2. SPR has also been used to measure it. The results are compared with those of the polylysine–peptide interaction, and they are discussed in relation to CK2 stimulation.

MATERIALS AND METHODS

Materials

CK2 was purified from bovine brain following essentially the method of Alcazar et al. [22]. Poly-L-lysine–agarose was used instead of phosvitin–Sephadex. The method of Towbin et al. [23]

Abbreviations used: SPR, surface plasmon resonance; CK2, protein kinase CK2; pKA, cAMP-dependent protein kinase; MAP1, microtubule-associated protein 1.

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was used to characterize further the enzyme by immunoblotting. The first antibody was generously given by Professor Michael Dahmus, Section of Molecular and Cell Biology, University of California, Davis, CA, U.S.A. The enzyme was assayed at 37 °C and pH 7.2, using the synthetic peptide RRREEETEEE as substrate, in a buffer composed of 20 mM Tris, 2 mM EGTA and 1 mM dithiothreitol. Where indicated, different NaCl concentrations were used. The assays were stopped by spotting aliquots on P81 phosphocellulose paper [24]. The papers were washed with 1 % phosphoric acid, and ethanol/diethyl ether (1:3, v/v) for the last washing. The peptide RRREEETEEE was synthesized by using a commercial peptide synthesizer (Applied Biosystems model 430A). HPLC was used as the last purification step. Poly-L-lysine was from Sigma. Its total molar concentration was obtained from the mg/ml concentration, using the number-average molecular mass (21 146 according to the manufacturer).

SPR

Plasmon resonance was attained basically using the technique originally proposed by Kretschmann and Raether [25]. Monochromatic light is directed at an incident angle upon an interface formed by a prism and a gold film. The surface electrons, at the other interface formed by the metal and the external medium, are then excited into a collective oscillation, termed 'plasmon'. Energy from the incident beam is then absorbed, giving rise to a diminution in the intensity of the reflected beam. The incident angle at which this resonance occurs (resonance angle) is experimentally observed as a minimum in the curve of intensity of light reflected versus the incident angle. The resonance angle is extremely sensitive to the refraction index of the adjacent external medium. Therefore the binding of proteins at the metal surface, or the binding of a ligand to an immobilized protein on this surface, changes the resonance angle. In practice, a constant angle close to the resonance angle is selected, and then changes in the refraction index produced by protein–ligand interactions are detected as changes in the intensity of light reflected [26].

A 15 mW helium/neon laser was used as source of p-polarized monochromatic light of 632.8 nm. A flow cell containing the gold surface was positioned on a rotary table. All the system was inside an isolated thermostatically controlled chamber, together with all running solutions and a peristaltic pump. The intensity of the light reflected was monitored by a silicon photodiode. A BASIC program was used to collect data and transfer them to a personal computer in the form of reflectance as a function of real time. Metal coatings of gold (45–50 nm thick) were prepared by evaporation on glass slides. The gold-coated slides were then stuck to the prism by an index-matching oil [thiodi(ethylene glycol)], which has a refractive index n_D of 1.52. The linear relationship between reflectance at a constant angle and the resonance angle was checked by registering both parameters for several water/methanol solutions having a range of refractive indices from 1.3331 to 1.3397. CK2, RRREEETEEE peptide and polylysine were immobilized by direct adsorption on to the gold surface [27]. A solution in running buffer was allowed to flow through the sample cell. The adsorption on to the gold film was continuously monitored by the increasing reflectance. Once the magnitude of this reflectance had levelled off, denoting surface saturation, running buffer was passed through before allowing the ligand to flow through. Unless otherwise indicated, the running buffer was composed of 20 mM Tris, 20 mM NaCl and 2 mM EGTA, pH 7.2. All measurements were carried out at 37 °C. Values following the $\pm$ symbol represent the S.E.M. of the data as obtained from a linear-regression fitting. The ligands adsorbed on the gold surface were stable, as denoted by the

stability of the reflectance signal under running buffer. Further evidence of stability was obtained with CK2 using a ^{32}P -phosphorylated preparation of enzyme. All radioactivity was found on the gold surface after polylysine binding, thus indicating no loss of adsorbed protein. The amount of CK2 adsorbed on to the gold surface could be estimated to be about 800 ng.

Kinetic analysis

The simplest mechanism able to describe the reversible interaction between an immobilized ligand (L) and a running ligand (P) is:

The corresponding kinetic equation is:

$$v = d\Gamma(\text{PL})/dt = k_1\Gamma(\text{L})[\text{P}] - k_{-1}\Gamma(\text{PL}) \quad (2)$$

where $\Gamma(\text{PL})$ and $\Gamma(\text{L})$ stand for the surface concentration of PL and free L respectively, and $[\text{P}]$ stands for the concentration of the running ligand. According to eqn. (1):

$$\Gamma(\text{L}) = \Gamma_t(\text{PL}) - \Gamma(\text{PL}) \quad (3)$$

where $\Gamma_t(\text{PL})$ stands for the total surface concentration of PL complex. The increase in reflectance signal, R , is proportional to the surface concentration of complex PL [26,28]:

$$R = \gamma\Gamma(\text{PL}) \quad (4)$$

$$R_t = \gamma\Gamma_t(\text{PL}) \quad (5)$$

where R_t stands for the maximal increase in reflectance, corresponding to the surface saturation by PL complex. Substituting eqns. (3), (4) and (5) into eqn. (2) yields the following expression for the rate of reflectance change:

$$dR/dt = k_1R_t[\text{P}] - (k_1[\text{P}] + k_{-1})R \quad (6)$$

P is constantly supplied by the flow. Therefore its free concentration may be considered constant and equal to the total present in the solution flowing into the cell. Under these pseudo-first-order conditions, a plot of dR/dt versus R must give a straight line. A secondary plot of the slopes $(k_1[\text{P}] + k_{-1})$ versus $[\text{P}]$, yields a second straight line from which both rate constants, k_1 and k_{-1} , can be evaluated. A more complex mechanism has been needed to describe the polylysine–peptide interaction. It is essentially a modification of the basic one described here. Its description can be found in the Appendix.

RESULTS

Polylysine–peptide interaction

Peptide as running ligand

Figure 1 shows the increasing reflectance due to the interaction of the RRREEETEEE peptide with polylysine immobilized on the gold surface. The much smaller signal due to the adsorption of peptide to the bare gold surface was also registered as a control (Figure 1 inset). When the peptide solution flowing through the cell was replaced by running buffer, the signal, R , did not decrease. It remained essentially constant for at least 2 h, thus indicating that the interaction process between peptide and polylysine is irreversible. The interaction process fits well to pseudo-first-order kinetics. Figure 2(a) shows the fitting of the data according to eqn. (6) for different peptide concentrations. When the slopes obtained from the pseudo-first-order kinetics were plotted against the peptide concentration, a straight line was obtained (Figure 2b). This indicates that the polylysine–

Figure 1 RRREEETEE peptide binding to polylysine immobilized on the gold surface

After immobilizing polylysine on the gold surface, a peptide solution was allowed to flow through the sample cell. The different solutions injected are indicated by numbers: 1, polylysine at 0.1 mg/ml; 2, running buffer; 3, peptide at 0.05 mg/ml; 4, running buffer. An inset scaled as for the full Figure shows the increase in reflectance obtained when the same peptide solution was allowed to flow over the bare gold surface without polylysine: 1, peptide at 0.05 mg/ml; 2, running buffer.

Figure 2 Kinetic analysis of the binding of peptide to polylysine

(a) Rate of reflectance increase versus reflectance according to eqn. (6) for different peptide concentrations (mg/ml): 1, 0.025; 2, 0.05; 3, 0.1; 4, 0.5. (b) Secondary plot of slopes versus peptide concentration.

peptide interaction fits well the model described above, according to which the rate of interaction would be of first-order for both peptide and polylysine. The intercept of the secondary plot is close to zero. Therefore the first-order constant for the complex dissociation, k_{-1} , is close to zero, in agreement with the observation that the interaction process is irreversible. The slope of the secondary plot yielded a second-order rate constant, k_1 , with value of $(3.15 \pm 0.04) \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$.

Figure 3 Polylysine binding to RRREEETEE peptide immobilized on the gold surface

After immobilizing peptide on the gold surface, a polylysine solution was allowed to flow through the sample cell. The different solutions injected are indicated by numbers: 1, peptide (0.05 mg/ml); 2, running buffer; 3, polylysine (0.005 mg/ml); 4, running buffer. An inset scaled as for the full Figure shows the increase in reflectance obtained when a polylysine solution (0.01 mg/ml) was allowed to flow over the bare gold surface without peptide: 1, polylysine.

Figure 4 Secondary plots for polylysine binding to immobilized peptide

(a) shows the secondary plot of slopes ($k_1[L] + k_{-1}$) versus polylysine concentration. (b) shows an inverse representation of the same data according to eqn. (8).

Polylysine as running ligand

The polylysine–peptide interaction can also be studied by allowing polylysine to flow over a peptide-coated gold surface. Figure 3 shows the increasing reflectance obtained when a polylysine solution was allowed to flow through the SPR cell after immobilizing peptide on the gold surface. The increase in reflectance was similar to that obtained by allowing peptide to flow over a polylysine-coated gold surface; in these cases also the interaction process was irreversible. The time course of reflectance also fitted well to pseudo-first-order kinetics according to eqn. (6). Figure 4(a) shows the corresponding secondary plot for different polylysine concentrations. As can be seen, the intercept goes practically to zero, according with the irreversibility

observed, but a strong deviation from linearity can be observed at high concentrations of polylysine.

A theoretical model for the polylysine–peptide interaction

Polylysine is a heterogeneous polymer. Its average molar concentration, however, can be obtained from the number-average molecular mass. Using this molecular mass, the second-order rate constant (k'_1) obtained from the extrapolation at low polylysine concentration in Figure 4 was $0.9 \times 10^6 \text{ M}^{-1} \cdot \text{min}^{-1}$. This constant decreases with the polylysine concentration, becoming $6.8 \times 10^4 \text{ M}^{-1} \cdot \text{min}^{-1}$ for the highest polylysine concentration used. It is noteworthy that the second-order rate constants obtained when polylysine was the running ligand are much larger than that obtained when the running ligand used was peptide ($k_1 = 3.15 \times 10^3 \text{ M}^{-1} \cdot \text{min}^{-1}$). This difference suggests a stoichiometry for the peptide–polylysine interaction different from the simple 1:1. Accordingly we made a modification to the model described in the Materials and methods section in order to take into account a different stoichiometry, the deviation from linearity of the secondary plot (Figure 4a) and the irreversibility of the interaction. If polylysine binds on average n molecules of peptide per molecule of polycation, the following equation (eqn. 6A) should be used instead of eqn. (6) when polylysine is the running ligand (see the Appendix):

$$dR/dt = nk_1 R_1[L] - nk_1[L]R \quad (6A)$$

According to the above equation, a secondary plot should yield a straight line. The intercept will be zero and the slope will be nk_1 . Therefore the second-order rate constant, k'_1 , obtained when polylysine is the running ligand, must be equal to nk_1 , k_1 being the second-order rate constant obtained by using peptide as the running ligand. The quotient k'_1/k_1 then yields the average number of peptide molecules bound per mol of polylysine.

As mentioned above, the secondary plot obtained when polylysine was the running buffer suffers a strong deviation from linearity at high polylysine concentration. A plausible explanation would be that the average number of peptide molecules bound per mol of polylysine, n , decreases upon increasing the polycation concentration. Polyelectrolytes change from a random-coil conformation at high ionic strength to a fully extended rod in the complete absence of salts [29]. Polylysine stimulation of CK2 activity occurs at moderately low ionic strength (within the range of approx. 0.05–0.1). It is conceivable that, under these conditions, polylysine acquires a rather extended conformation, with a more pronounced susceptibility to intermolecular interactions [30]. Increasing the polycation concentration must then increase these intermolecular interactions and restrict the peptide binding stoichiometry. Accordingly we tried the following equation to express the diminution of n with increasing polylysine concentration:

$$n = n_0 / (1 + \kappa[L]) \quad (7)$$

where n_0 stands for the value of n when $[L]$ becomes zero, and κ is an empirical constant. Using eqn. (7), eqn. (6A) becomes:

$$dR/dt = [n_0 / (1 + \kappa[L])] k_1 R_1[L] - [n_0 / (1 + \kappa[L])] k_1 R[L]$$

The equation corresponding to the secondary plot is now:

$$\text{Slope} = n_0 k_1 [L] / (1 + \kappa[L])$$

and taking inverses:

$$1/\text{Slope} = \kappa / n_0 k_1 + (1/n_0 k_1)(1/[L]) \quad (8)$$

A plot of $1/\text{slope}$ versus $1/[L]$ (Figure 4b) must then yield a straight line from which κ and n_0 can be obtained. The n_0 and κ

Figure 5 Polylysine dependence of CK2 activity

■, Experimental enzyme-activity data. A peptide solution (0.5 mg/ml) was incubated with the indicated polylysine concentrations in running buffer at 37 °C. After 15 min, the assays were initiated by simultaneously adding enzyme, ATP (0.1 mM) and MgCl_2 (5 mM). The phosphate incorporated into peptide was determined as described in the Materials and methods section. The continuous curve is theoretical. It is obtained by using eqns. (9), (11) and (12) and the kinetic data of Table 1.

Table 1 Effect of polylysine on the kinetic constants for the RRREEETEE peptide phosphorylation by CK2

The enzyme was assayed as described in the Materials and methods section in a buffer composed of 20 mM Tris, 2 mM EGTA, 20 mM NaCl, 5 mM MgCl_2 and 0.1 mM ATP. Albumin (0.1 mg/ml) was added to avoid surface denaturation. Polylysine was 0.1 mg/ml when present.

	$V_{\max}$ (nmol of P/min per mg)	K_m (mg/ml)
Control	185 ± 17	0.07 ± 0.013
+ Polylysine	1170 ± 130	0.12 ± 0.02

values obtained were 324 ± 13 and $108 \pm 19 \text{ (mg} \cdot \text{ml}^{-1})^{-1}$ respectively.

CK2 activity stimulation by polylysine

Figure 5 shows the activity of CK2 as a function of polylysine concentration. As can be observed, a strong stimulation takes place from $1 \mu\text{g/ml}$ to 0.1 mg/ml polylysine. Assuming that this enzyme stimulation is substrate-mediated, the interaction between polylysine and the peptide substrate would produce a new substrate on which the enzyme would act with a higher efficiency. The enzyme activity would then be given by:

$$v = V_{\max}[P]/(K_m + [P]) + V'_{\max}[P']/(K'_m + [P']) \quad (9)$$

where $V_{\max}$, K_m , $V'_{\max}$ and K'_m stand for the maximal velocity and Michaelis constants of the enzyme acting on peptide and on the peptide–polylysine complex respectively. These kinetic constants were determined in a separate experiment (Table 1). $[P]$ and $[P']$ stand for the concentration of peptide and peptide complexed by polylysine, both in mg/ml. The rate at which P reacts with polylysine to form P' will be given by:

$$d[P]/dt = -nk_1[P][L] \quad (10)$$

Once k_1 and the stoichiometry of the interaction with polylysine, n , are known, $[P]$, and $[P']$ can be calculated by integration of eqn. (10):

$$[P'] = \frac{n[P]_0[L]_0\{\exp[k_1t([P]_0 - n[L]_0)] - 1\}}{[P]_0\exp[k_1t([P]_0 - n[L]_0)] - n[L]_0} \quad (11)$$

$$[P] = [P]_0 - [P'] \quad (12)$$

Using eqns. (11) and (12) to calculate $[P']$ and $[P]$, eqn. (9) gives the rate of peptide phosphorylation for different polylysine concentrations. These calculated values have been included in Figure 5 (continuous line) in order that they may be compared with the experimental activity obtained at the same polylysine concentrations (■). As can be seen, the activity calculated agrees well with that measured under the same temperature, ionic strength and pH at which the SPR measurements were carried out.

The results described above strongly support the hypothesis that CK2 stimulation by polylysine is due to an interaction of the substrate with the polycation, giving rise to a complex which seems to be a better substrate for the enzyme. The extent of CK2 stimulation by polylysine depends on ionic strength.

Figure 6 Effect of NaCl on the binding of peptide to polylysine and on the extent of CK2 activity stimulation by polylysine

(a) Shows the time course of reflectance corresponding to the flowing of peptide (0.05 mg/ml) on polylysine immobilized on the gold surface at three NaCl concentrations: 1, 250 mM; 2, 150 mM; 3, 20 mM. (b) Shows the percentage of activity stimulation by polylysine (0.003 mg/ml) at the indicated NaCl concentrations (■) and the percentage of reflectance increase obtained from flowing peptide on polylysine at the indicated NaCl concentrations (○).

Figure 7 Electrophoretic evidence for an RRREETEEE–polylysine complex

Peptide solutions were filtered through an Amicon membrane (30 kDa nominal cut-off) in the absence or in the presence of the indicated polylysine concentrations. Peptide solutions that had not been filtered were run as controls. (a) Peptide at 0.5 mg/ml: lanes 1, 4 and 7 are controls; lanes 2, 5 and 8 are the retained solutions; lanes 3, 6 and 9 are the filtered solutions. (b) Peptide at 0.15 mg/ml: lanes 1 and 3 are controls; lanes 2 and 4 are the filtered solutions. (c) 0.5 mg/ml peptide solutions in the presence of the indicated concentrations of polylysine or spermine were filtered as in (a): lanes 3, 4, 7 and 8 are the retained solutions; lanes 1, 2, 5 and 6 are the filtered solutions.

Maximal stimulation is attained when ionic strength is minimal. The stimulation decreases with increasing ionic strength, and it is almost negligible when the ionic strength reaches 0.15–0.2. Additional evidence supporting the notion that CK2 stimulation is substrate-mediated was obtained by studying the effect of ionic strength on peptide–polylysine interaction. Figure 6(a) shows this effect on the reflectance signal obtained, using peptide as the running ligand on a polylysine-coated gold surface. Maximal reflectance decreases when the ionic strength increases. Upon increasing ionic strength, a close parallelism between the percentage of enzyme stimulation and the percentage of maximal reflectance obtained could be observed (Figure 6b).

According to the stoichiometry evaluated, the peptide–polylysine interaction must give rise to a high-molecular-mass complex, and this was confirmed by ultrafiltration. Peptide solutions were filtered through an Amicon membrane with nominal 30 kDa-molecular-mass cut-off. The filtered and retained solutions were then analysed by SDS/PAGE (Figure 7). In the absence of polylysine, a small amount of peptide was retained by the membrane, but most of the peptide passed through the membrane. In the presence of polylysine, an increasing amount of peptide could be found in the retained solutions, and a concomitantly lower amount of peptide passed through the membrane (Figures 7a and 7b). In contrast with polylysine, spermine, which is also known to stimulate CK2 activity, did not help to retain the peptide. A peptide solution in the presence of spermine passed through the membrane, whereas the peptide in the presence of polylysine was found mostly in the

Figure 8 Binding of polylysine to CK2

(a) The enzyme was allowed to flow at 0.007 mg/l in a high-ionic-strength buffer (about 0.6 M NaCl). Once the signal had levelled off, running buffer was injected. A polylysine solution (0.01 mg/ml) in running buffer was then allowed to flow over the immobilized CK2 (the arrow indicates the injection of polylysine). An inset scaled as for the main Figure shows the injection of the same polylysine solution over the bare gold surface. (b) Pseudo-first-order constant corresponding to the initial phase of the binding as function of the polylysine concentration.

Figure 9 Experimental, and theoretical polylysine stimulation of CK2 activity

(a) The enzyme was assayed in the presence of the indicated polylysine concentrations as described in the legend of Figure 5 (■). The continuous curve is theoretical and has been obtained by using eqns. (13), (14) and (15) and the data of Table 1. The data of Figure 5 have also been included in (b) for comparison with the fitting in (a).

retained solutions (Figure 7c). This is in agreement with the fact that no peptide-spermine interaction able to alter the aggregation state of the peptide could be observed by SPR.

CK2-polylysine interaction

The binding of polylysine to CK2 was also studied by SPR. Figure 8(a) shows the increase in reflectance when polylysine was allowed to flow through the SPR cell, which contained CK2 immobilized on the gold surface. Similarly to the case of peptide-polylysine interaction, the binding of polylysine to CK2 was irreversible. No dissociation could be observed after a buffer washing of about 7 h. As deduced from the time course of reflectance, the interaction seems to be very slow. The signal started to level off after several hours of polylysine flow. The CK2 activity stimulation by polylysine, however, seems to be much faster. In fact it is observed to be maximal after a few minutes of incubation of polycation with the phosphorylation reaction mixture.

As Figure 8(a) shows, the increase in reflectance seems to have a biphasic nature. After an initial increase, the reflectance signal went on increasing in a second, slower, phase. Kinetic analysis yielded an initial phase, with a rate constant, ke_1 , of 0.037 min⁻¹, and a second phase with a very low rate constant, ke_2 , of 3.3×10^{-4} min⁻¹. The first phase of the reaction seems to depend on the polylysine concentration (Figure 8b). The second phase, however, yielded the same rate constant when the polylysine concentration was changed; and substitution of the polylysine solution flowing through the cell during this second phase by running buffer did not prevent the reflectance from increasing at the same rate.

The results described above suggest a two-step mechanism for the interaction of CK2 with polylysine. In the first step the polycation would bind the enzyme with a second-order rate constant, ke_1 , of 7.9×10^4 M⁻¹·min⁻¹. Once the polylysine-CK2 complex has been formed, it would evolve to a different conformation in a slow, first-order process with a half-life of about 30 h. The full process is too slow to explain the stimulation of CK2 activity as a direct consequence of the binding of polylysine to the enzyme. However, there exists the possibility that CK2 activity stimulation may follow the initial faster step of binding of polylysine to CK2. If this were the case, the enzyme activity in the presence of polylysine would be given by:

$$v = k_{\text{cat}}[E][S]/(K_m + [S]) + k'_{\text{cat}}[E'][S]/(K'_m + [S]) \quad (13)$$

where k_{cat} , K_m , k'_{cat} , and K'_m stand for the catalytic and Michaelis constants of the free and polylysine-complexed enzyme respectively. $[E]$ and $[E']$ stand for the concentration of free and complexed enzyme respectively. Both concentrations can be evaluated from the rate constant for the initial binding step obtained from the data in Figure 8(a) ($ke_1 = 7.9 \times 10^4$ M⁻¹·min⁻¹):

$$[E] = [E_0]\exp(-ke_1[L]t) \quad (14)$$

$$[E'] = [E_0] - [E] \quad (15)$$

where $[L]$ stands for the several polylysine concentrations used to stimulate the enzyme. Eqn. (13), together with eqns. (14) and (15), yield the enzyme activity as a function of the polylysine concentration. The results are plotted in Figure 9(a) together with the experimental data for activity stimulation. It can be seen that, in spite of using the faster phase of the polylysine interaction with CK2, the enzyme-activity stimulation does not follow the polylysine binding to the enzyme. Figure 9(b) shows the fitting of the experimental stimulation to the peptide-polylysine interaction. Comparison with Figure 9(a) indicates that, although polylysine binds both enzyme and substrate, the enzyme activity stimulation seems to follow the polylysine binding to the peptide rather than to the enzyme.

DISCUSSION

The results presented here show that polylysine interacts with the peptide substrate of CK2, RRREEETEEE and also with the enzyme itself. In both cases the interaction seems to be irreversible, meaning that no dissociation constant, k_{-1} , could be measured under the same experimental conditions under which the interaction process occurs. The data for the interaction with peptide are consistent with a variable stoichiometry according to which the number of peptide molecules bound per mol of polylysine decreases with increasing polylysine concentration. When the kinetic parameters of the enzyme, together with the binding kinetic constant, and the variable stoichiometry of the peptide–polylysine interaction are used to calculate the activity as a function of the polylysine concentration, the results agree well with the experimental data for enzyme stimulation, explaining the low concentration of polylysine needed to stimulate the enzyme. A good correlation also exists between the influence of ionic strength on the peptide–polylysine interaction and on the polylysine stimulation of enzyme activity. As Figure 6 shows, the maximal reflectance registered from the peptide–polylysine interaction decreases with the NaCl concentration. This result suggests that the inhibitory effect of NaCl on the polylysine stimulation of CK2 is due to an inhibition of the peptide–polylysine interaction at high NaCl concentration, and it is in agreement with the results shown in Figure 5, supporting a substrate-mediated mechanism of polylysine stimulation.

The binding of polylysine to CK2 has already been reported [12,13]. This binding affects the enzyme conformation, giving rise to high-molecular-mass complexes [13]. As far as we know, however, no data concerning the kinetics or equilibrium constant of the interaction have been reported. Our SPR data show that the binding of polylysine to CK2 has a biphasic nature. After an initial phase with a rate constant dependent on the polylysine concentration, a very slow second process can be registered that is independent of the polylysine concentration, as it would be for a conformational change triggered by the polylysine binding. The full process appears to be irreversible. The low rate of the binding process, as compared with the rate at which stimulation is observed, together with the irreversibility of the binding, make polylysine hardly conceivable as reproducing *in vitro* the action of a natural polylysine-like regulator acting directly on the enzyme as a second-messenger-type regulator. Taken together, the results presented here support a mechanism of CK2 stimulation by polylysine based on the interaction between substrate and polycation. This interaction gives rise to an aggregation of peptide on which the enzyme would act with a higher $V_{\max}$.

Polyamines and basic polypeptides share the capability of activating CK2. The mechanism whereby polyamines exerts the stimulation, however, seems to differ from that of basic polypeptides, as indicated by many observations [2]. The reports concerning the binding of spermine to CK2 [31] and the spermine-induced stabilization of a particularly active conformation of the enzyme [32], strongly support that enzyme activation is a direct consequence of the spermine binding to CK2. In agreement with this, we do not find any peptide–spermine interaction of the type reported here for polylysine. It seems that both polyamines and basic polypeptides interact with CK2. The polyamine interactions would give rise to a more active enzyme, whereas in the case of basic polypeptides the stimulation of enzyme activity does not follow this interaction but is a consequence of conformational changes induced on the phosphorylatable substrate.

The results presented here are in agreement with previous observations concerning the phosphorylation of physiological and non-physiological CK2 protein substrates. The polylysine-

induced stimulation of the enzyme has been found to parallel a polylysine-induced aggregation of all substrates tested [20]. The results for the polylysine-dependent phosphorylation of calmodulin also support the notion of a substrate-mediated mode of stimulation. It has been demonstrated by ^1H NMR that polylysine interacts with calmodulin and that this interaction causes aggregation of this protein [33]. CK2 may undergo large conformational changes, as an associating–dissociating system, particularly influenced by ionic strength [32,34]. It has been suggested that this oligomeric variability may form part of the regulatory system of the enzyme [32]. Within this context it is noticeable that polylysine stimulation is maximal at the lowest attainable ionic strength, where the enzyme becomes aggregated, decreasing its catalytic activity. This suggests the possibility that polylysine-induced structural changes in CK2 substrates may mimic *in vitro* some naturally induced changes in the CK2 substrates which would modulate the enzyme activity, relieving the down-regulation imposed by the conformational changes of the enzyme and therefore contributing to its mechanism of regulation. Similarly, the observation that polylysine inhibits the phosphorylation of the β -subunit of the enzyme, but stimulates that of a nonapeptide reproducing the phosphoacceptor site on β -subunit, has led to the suggestion that naturally occurring compounds may play a role in determining the targeting of definite substrates, bringing them to a form having a higher susceptibility to phosphorylation [18].

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APPENDIX

If polylysine binds on average more than one molecule of peptide per molecule of polycation, we have:

Assuming that LP_n is formed by a simple sequence of bimolecular interactions:

and that the steady-state approximation is applied for the intermediates LP_i ($i < n$), the rate of initiation of the sequence must be equal to the rate of termination [1]:

$$d[LP_n]/dt = k_n[P][LP_{n-1}] = k_1[P][L]$$

Accordingly, when peptide is used as the running ligand, P, flowing over a polylysine-coated gold surface, the rate of the irreversible LP_n formation would be:

$$v = d\Gamma(LP_n)/dt = k_1\Gamma(L)[P] \quad (A2)$$

According to the stoichiometry defined by eqn. (A1), eqn. (3) of the main paper can be substituted in eqn. (A2) to yield:

$$v = d\Gamma(LP_n)/dt = k_1[\Gamma_t(LP_n) - \Gamma(LP_n)][P]$$

and using eqns. (4) and (5) of the main paper:

$$dR/dt = k_1R_t[P] - k_1[P]R \quad (A3)$$

According to this equation, a secondary plot must yield a straight line, the intercept of which is zero, and the slope is k_1 , in

agreement with the results obtained using peptide as the running ligand.

Using polylysine as the running ligand, on a peptide-coated gold surface, the rate of LP_n formation will be:

$$v = d\Gamma(LP_n)/dt = k_1\Gamma(P)[L] \quad (A4)$$

where $\Gamma(P)$ stands for the surface concentration of P, and $[L]$ stands for the running polylysine concentration. According to the stoichiometry defined by eqn. (A1), the surface concentration of P will be:

$$\Gamma(P) = [\Gamma_t(LP_n) - \Gamma(LP_n)]n$$

and the rate of LP_n formation:

$$v = d\Gamma(LP_n)/dt = k_1[\Gamma_t(LP_n) - \Gamma(LP_n)]n[L] \quad (A5)$$

Using again eqns. (4) and (5) of the main paper, eqn. (A5) becomes:

$$dR/dt = nk_1R_t[L] - nk_1[L]R \quad (A6)$$

The constant obtained from a secondary plot, when polylysine is the running ligand, should be then nk_1 .

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