Chemical Features of the Protein Kinase CK2 Polyamine Binding Site[†,‡]

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ABSTRACT: Protein kinase CK2 is a ubiquitous eukaryotic Ser/Thr kinase whose catalytic activity is enhanced several times by polyamines. We have shown previously that the regulatory β-subunit of CK2 bears a polyamine binding site located in the region Asp51−Tyr110. In the present study, we have used spermine analogs to investigate the structural requirements of the CK2 polyamine binding site. We have observed a strong correlation between the stimulations of CK2 activity by all tested polyamines and their binding efficiencies to the enzyme. As a result, spermine was found to be the most efficient stimulator of the kinase activity and the best CK2 ligand. The effect of the pH on the stimulation of CK2 activity by spermine strongly suggests the involvement of ionic interactions between the positive charges of spermine and the negative charges of acidic amino acids of the β-subunit. Using a fusion protein made of MBP and the β-subunit region encompassing amino acid residues Asp51−Pro110, we have studied the binding of spermine as a function of the ionic strength. We show that this region delineates a functional and autonomous domain containing a binding site involved in the interaction with the four positive charges of spermine. Altogether, these results led to the elaboration of the first model defining the crucial structural parameters of a polyamine−protein interaction at the molecular level.

The protein kinase CK2 (CK2) is a serine/threonine protein kinase expressed in a wide range of eukaryotic organisms from yeast to human. The enzyme is present in the cytoplasm and the nucleus of a number of cells (Hathaway & Traugh, 1982; Krebs et al., 1988) and is composed of three dissimilar subunits, i.e. the catalytic subunits of 35−44 kDa (Padmanabha & Glover, 1987) and the β-subunit of 24−29 kDa. These subunits associate to generate native structures exhibiting the stoichiometry $\alpha_2\beta_2$, $\alpha'_2\beta_2$, and $\alpha\alpha'\beta_2$. The β-subunit is self-phosphorylated and is considered as a regulatory element of the native enzyme being able to stimulate by 5−10-fold the α-subunit activity (Cochet & Chambaz, 1983a; Traugh et al., 1990; Filhol et al., 1991b). A number of CK2 substrates are transcription factors and oncoproteins such as Myc (Lüscher et al., 1989), Myb (Krebs et al., 1988; Lüscher et al., 1990), Fos (Carroll et al., 1988), and the antioncogene p53 (Meek et al., 1990). Other substrates were found to be cytoplasmic or membrane-associated proteins. CK2 recently became a target of interest when the knockout of the gene coding for its catalytic subunit in yeast was shown to be lethal (Padmanabha et al., 1990).

However, until now, no intracellular messenger involved in CK2 regulation has been identified. *In vitro* experiments unambiguously demonstrated that naturally occurring polyamines are strong CK2 activators (Cochet & Chambaz, 1983b). In fact, the stimulation of CK2 catalytic activity by polyamines is detected with acidic substrates like casein and is strictly dependent on the presence of the β-subunit (Filhol et al., 1991a) and a low magnesium concentration (Hathaway & Traugh, 1984). With such substrates, it is worth noting that, in the absence of polyamines, a 20−30 mM magnesium concentration is required for maximal CK2 activity. This concentration is 4−5 times higher than that necessary to obtain full activation of the α-subunit alone and most of the other protein kinases. Thus, the intracellular magnesium concentration, estimated to be around 1 mM, is far from the optimum concentration required to support a maximal CK2 activity *in vitro*. Our working hypothesis therefore is that polyamines may be considered as potential intracellular modulators of CK2 activity, acting in synergy with magnesium. The spermine binding activity of oligomeric CK2 was shown to be borne by its β-subunit, and a Scatchard plot analysis disclosed that the enzyme has two binding sites with different affinities (Filhol et al., 1991a).

Polyamines are ubiquitous cellular components which are indispensable for cell proliferation and differentiation (Pegg, 1986; Scalabrino et al., 1991). Interestingly, several enzymatic activities, including insulin receptor phosphorylation, vitamin D receptor activity, GTPase activity of G proteins, and protein phosphatase 2A and topoisomerases I/II activities, have also been shown to be modulated by polybasic molecules (Xu et al., 1991; Morishima et al., 1994; Bueb et al., 1992; Tung et al., 1985; Srivenugopal & Morris, 1985; Pommier et al., 1989). Despite the crucial roles played in the cell by most of these polyamine-stimulated enzymes, as

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[‡] This work is dedicated to the memory of Serge Pares, who died tragically this year in an air crash.

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yet, no accurate mechanism has been proposed to explain the structural effect of these polybasic molecules on their target proteins at the molecular level. Nevertheless, interaction of polybasic compounds with nucleic acids has been analyzed by a number of groups. Spermine analogs that differ in the number of methylenes separating the positively charged amino and imino groups have been successfully used to investigate the effect of polyamines on the DNA structure (Basu et al., 1989, 1992, 1993; Edwards et al., 1991; Delcros et al., 1993; Thomas & Thomas, 1993; Garriga et al., 1993). The ionic charge, pH, and ionic concentration of the medium are parameters that govern the interaction of polyamines with the duplex DNA. In the presence of polyamine analogs, the induction and stabilization of left-handed Z-DNA are highly dependent on the ligand molecule structure (Thomas & Thomas, 1993). Following the observation that protein−nucleic acid interaction is highly salt-dependent, Record et al. have shown that polycation-nucleic acids interactions shared the same characteristics (Record et al., 1976a). With respect to these results, Mascotti and Lohman have applied a useful method to determine the number of ionic interactions formed in a complex between polybasic molecules and a linear nucleic acid (Mascotti & Lohman, 1990, 1992, 1993).

Using a photoaffinity labeling method, we have recently identified a spermine binding site located in an acidic stretch of the CK2 β-subunit (Leroy et al., 1995). As a working hypothesis, we have proposed a structural model taking into account the mapping results in which both glutamic acid residues 73 and 77 could interact with two of the four positive charges of spermine. In the present study, we have characterized the interaction between spermine and the polyamine binding domain of CK2. Using a series of polyamine analogs, we provide evidence that the stimulation of CK2 activity by a polyamine molecule and its binding efficiency toward CK2 rely not only on its number of positive charges but also on the length of its aliphatic chain. Subsequent analyses of the effects of pH and of sodium chloride concentration allowed us to determine the chemical nature and the number of β-subunit amino acid residues involved in the interaction. In this view, our results incited us to elaborate a new model defining the positions of the polyamine positive charges toward four negative charges present in the acidic stretch of the β-subunit.

EXPERIMENTAL PROCEDURES

Materials. Polyamine analogs were generous gift from Dr. B. G. Feuerstein (Department of Pediatrics, School of Medicine, University of California, San Fransisco, CA 94143) and the Marrion Merrell Dow Research Institute (Cincinnati, OH).

[^{3}H]spermine (60 Ci/mmol) was purchased from Isotopchim (France).

Expression and Purification of Drosophila CK2. Insect cells (10^6 Sf9 cells/mL) were coinfected with EV 55 Dm α and EV 55 Dm β viruses at a multiplicity of infection of 5−10 as previously described by Filhol et al. (1991b). The cell lysate was sonicated for 3 min and centrifuged at 100000*g* for 30 min. The soluble extract was diluted in buffer A [10 mM Tris-HCl (pH 7.5)/1 mM dithiothreitol/1% glycerol/0.1% Triton X-100] to a final concentration of 0.2 M NaCl, applied onto a phosphocellulose column previously equilibrated in buffer A, and recycled three times through the column. A 0.2 to 1.5 M linear NaCl gradient in buffer A was applied. The fractions containing CK2 activity were pooled and concentrated on a XM 50 membrane (Amicon) to 90 mL. The concentrated solution was diluted five times in buffer B [10 mM Tris-HCl (pH 7.5)/1 mM DTT/1% glycerol] and loaded onto a heparin-Sepharose column previously equilibrated in the same buffer. A 0.4 to 1.2 M NaCl linear gradient in buffer B was developed. Fractions containing CK2 were pooled and concentrated on a PM 30 membrane (Amicon). The concentrated solution was diluted ten times in buffer B and loaded onto a DEAE-cellulose column previously equilibrated in the same buffer. A 0 to 1 M linear NaCl gradient in buffer B was applied. The fractions containing CK2 were pooled and stored at −80 °C in 1 M NaCl until they were used.

Expression and Purification of the β-Subunit Domain Encompassing Residues Asp51−Pro110 (MBPβ51−110). The domain encompassing residues Asp51−Pro110 of the chicken β-subunit was expressed in *Escherichia coli* as a fusion protein with the maltose binding protein (MBP)[1] (di Guan et al., 1988; New England BioLabs). The sequence coding for the β-subunit domain (representing amino acids 51−110) was amplified by PCR from the corresponding cDNA (kindly provided by Dr. E. Nigg). The PCR amplification led to DNA fragments carrying *Bam*HI site *Hin*dIII sites located respectively at the 5′ and 3′ extremities of the coding sequence. PCR products were cloned in the pMAL-C2 vector (protein fusion and purification system, New England BioLabs). The resulting recombinant vector was used to transform *Escherichia coli* strain BL21. The culture was induced during 2 h with 0.3 mM isopropyl thiogalactopyranoside (IPTG). The cell pellet was resuspended in cold lysis buffer [10 mM phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM EDTA, and 10 mM EGTA (pH 7.0)]. After a thermal shock (−70 to 20 °C) and a 3 × 2 min sonication, the lysate was adjusted to 0.5 M NaCl and subjected to centrifugation at 9000*g* for 20 min. The supernatant was mixed with amylose resin (New England BioLabs) at 4 °C during 1 h, and the fusion protein was eluted with 10 mM maltose added to the column buffer [10 mM phosphate, 0.5 M NaCl, 1 mM sodium azide, and 1 mM EGTA (pH 7.0)]. The recombinant protein MBPβ51−110 was finally concentrated on a Centricon cell up to 2 mg/mL.

Binding of Spermine. Recombinant proteins (CK2 or the fusion protein MBPβ51−110) were incubated at 4 °C for 5 min with 0.5 μM [^{3}H]spermine (10^6 cpm) in the absence or presence of different concentrations of nonradioactive polyamines in a final assay volume of 80 μL of Tris buffer (10 mM Tris-HCl at pH 7.4). The NaCl concentration was set at 35 mM in the binding experiments (except where indicated). The mixtures were then rapidly centrifuged at 4 °C according to Penefsky (1977) through a small Sephadex G50 superfine column previously equilibrated in the Tris buffer containing 1 mg/mL BSA. Bound [^{3}H]spermine was determined by radioactive counting of the excluded volume, after subtraction of the blank values obtained in the absence

[1] Abbreviations: MBP, maltose binding protein; MBPβ51−110, fusion protein involving MBP and the β-subunit domain encompassing residues Asp51−Pro110; polyamines, systematic names and chemical formulas given in Table 1.

Table 1: Polyamine Analogs Used in the Present Study

abbreviation	trivial name	systematic name	chemical formula
spm(3-4-3)	spermine	1,12-diamino-4,9-diazadodecane	NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2
spd	spermidine	1,8-diamino-4-azaoctane	NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH_2
put	putrescine	1,4-diaminobutane	NH_2-$(CH_2)_4$-NH_2
Bespm	bis(ethyl)spermine	1,12-bis(ethylamino)-4,9-diazadodecane	C_2H_5-NH-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH-C_2H_5
3-3-3	norspermine	1,11-diamino-4,8-diazaundecane	NH_2-$(CH_2)_3$-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH_2
Be3-3-3	bis(ethyl)norspermine	1,11-bis(ethylamino)-4,8-diazaundecane	C_2H_5-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH-$(CH_2)_3$-NH-C_2H_5
Dadd		1,12-diaminododecane	NH_2-$(CH_2)_{12}$-NH_2
3-2-3		1,10-diamino-4,7-diazadecane	NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-NH-$(CH_2)_3$-NH_2
Be3-8-3		1,16-bis(ethylamino)-4,14-diazahexadecane	C_2H_5-NH-$(CH_2)_3$-NH-$(CH_2)_8$-NH-$(CH_2)_3$-NH-C_2H_5
Be4-4-4		1,14-bis(ethylamino)-4,10-diazatetradecane	C_2H_5-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH-C_2H_5
4-4-4		1,14-diamino-4,10-diazatetradecane	NH_2-$(CH_2)_4$-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2
4-4-4-4		1,19-diamino-5,10,15-triazanonadecane	NH_2-$(CH_2)_4$-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2

of recombinant protein. Blank values represent less than 0.2% of the input radioactivity.

Effect of the Ionic Strength on the Spermine Binding Efficiency. Record et al. have developed a general thermodynamic analysis for the effect of monovalent ions on the observed association constants (K_{obs}) of ligand−nucleic acid interactions (Record et al., 1976a,b). In their studies, log K_{obs} is a linear function of log[M^+] described by eq 1:

$$\log K_{obs}/\log[M^+] = -\Psi z \quad (1)$$

where M^+ is the monovalent counterion, z represents the number of charges on the ligand, and Ψ is the fraction of counterion bound in the thermodynamic sense per nucleic acid charge. To accurately determine the Ψ value, we can use the following equation:

$$\Psi = 1 - 2(\zeta)^{-1} \quad (2)$$

where

$$\zeta = e^2/\epsilon KTb \quad (3)$$

In eq 3, e represents the electronic charge, ϵ is the bulk dielectric constant, K is the Boltzman constant, T corresponds to the absolute temperature, and b is the distance between two consecutive phosphate groups on one strand of DNA. In the study of the polylysine−DNA complex, Ψ was determined to be 0.88, taking into account the b value which is equivalent to 3.4 Å in this case (Record et al., 1976a).

For the analysis of the spermine−β-domain interaction, the ratio $e^2/\epsilon KT$ may be considered unchanged because of the nature and temperature of the incubation medium. This ratio may be therefore written as the constant A in the following equation:

$$\zeta_{DNA} = A/3.4\ \text{Å}$$

In our working hypothesis, we consider the fact that the polyamine binding site is composed of acidic amino acid residues belonging to an α-helix and spaced by three residues in the β-subunit. In such a conformation, the b value is equivalent to 6 Å and the following equation may be written

$$\zeta_{\beta-domain} = A/6\ \text{Å} = \zeta_{DNA} \times 3.4\ \text{Å}/6\ \text{Å}$$

The resulting Ψ parameter is equivalent to $1 - 2(16.6 \times 3.4/6)^{-1} = 0.79$.

CK2 Activity Assay. Phosphorylation of casein by CK2 was performed as described by Cochet et al. (1981). Briefly, CK2 (1 nM) was incubated at 30 °C in the presence of casein (0.4 mg/mL), ATP (10 μM), [γ-^{32}P]ATP (10^6 cpm/assay), and $MgCl_2$ (1 mM) in the absence or presence of increasing concentrations of polyamine in a final volume of 80 μL. Phosphorylation reactions were stopped by the addition of 2 mL of trichloroacetic acid (12.5%), and 20 μL of casein (30 mg/mL) was added per assay. Casein was precipitated at 4 °C by centrifugation for 10 min at 3000*g*. The supernatant was removed, and the pellet was redissolved in 200 μL of NaOH (1 M). Two additional precipitation steps were performed, and the pellet redissolved following the third one was analyzed by liquid scintillation counting.

CK2 activity was calculated after subtraction of the blank values obtained in the absence of kinase.

Analysis of the pH effect on the stimulation of CK2 activity by polyamines has been performed in the presence of different buffers: acetate buffer (20 mM), pH 4.4; MES buffer (20 mM), pH 6.0; Tris buffer (20 mM), pH 7.4 and 9.0; and Caps buffer (20 mM), pH 10.4.

RESULTS

Interactions of Spermine Analogs with CK2. A number of studies has previously shown that CK2 phosphotransferase activity on acidic substrates is stimulated several times by polybasic compounds positively charged at physiological pH values. To determine whether the binding of polyamines to CK2 is only due to the ligand positive charges or is also dependent on the polybasic compound structure, we used spermine analogs bearing two to four positive charges and different aliphatic chains (Table 1) for the investigation of the polyamine−CK2 interaction. The spermine analogs were tested both on the phosphotransferase activity of CK2 in basal magnesium conditions (1 mM) and on the CK2−spermine binding activity. As disclosed in Figure 1, spermine was found to be the most efficient polyamine to stimulate CK2 activity. Spermine at a concentration of 1 mM stimulated CK2 activity by 7-fold. A 6-fold activation of the kinase activity was observed in the presence of 1 mM norspermine, bis(ethyl)norspermine, and bis(ethyl)spermine. Spermidine was found to be less efficient, leading to a 4.5-fold stimulation of CK2 activity. Be3-8-3, Be4-4-4, and putrescine were shown to be poor activators of the enzyme, exhibiting less than a 3-fold stimulation of the kinase activity. The data from Figure 1 were used to determine graphically the maximal activity and the analog concentration giving half-maximal stimulation of the kinase (Table 2). Maximal activities were 7.2 and 2.4 pmol/min for spermine and Be4-4-4, respectively. The corresponding A_{50} values were obtained for concentrations of 45 and 255 μM, respectively.

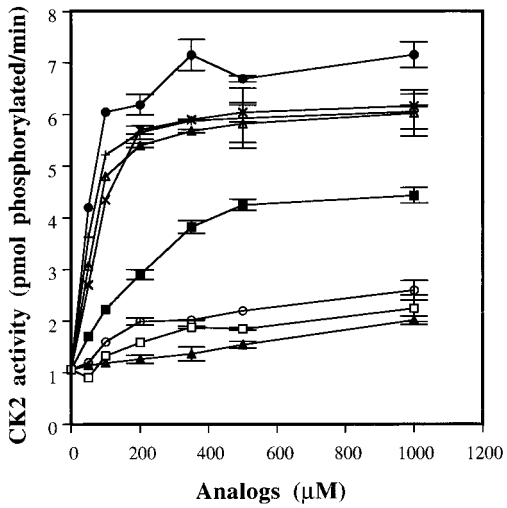

FIGURE 1: Effects of the polyamine analogs on CK2 activity. Aliquots of CK2 (3.6 nM) were incubated with 0.4 mg/mL casein, [γ-^{32}P]ATP (10^6 cpm/assay), 10 μM ATP, and 1 mM $MgCl_2$ at 30 °C for 8 min in the absence or presence of increasing concentrations of the following polyamines: spm (●), spd (□), put (▲), Bespm (×), 3-3-3 (+), Be3-3-3 (△), Be3-8-3 (○), and Be4-4-4 (■). CK2 activity was calculated as the number of ^{32}P picomoles incorporated per minute. Data are the results of assays performed in duplicate.

Table 2: Maximal Activation Efficiency and A_{50} of the Polyamine Analogs

	spm	3-3-3	Be3-3-3	Bespm	spd	Be3-8-3	Be4-4-4	put
max velocity (pmol/min)	7.2	6.0	6.0	6.2	4.6	3.0	2.4	–
A50 (μM)	45	45	60	75	185	185	255	–

[a] The maximal CK2 activity and the concentration of polyamine giving half-maximal activity were determined graphically from Figure 1.

In terms of activation efficiency of the polyamine analogs, the following order may be proposed: spermine > norspermine, bis(ethyl)norspermine, and bis(ethyl)spermine > spermidine > Be3-8-3, Be4-4-4, and putrescine.

These results lead to an important question. Could the most efficient polyamines for CK2 stimulation be the best CK2 ligands? To address this question, the different polyamine analogs were tested as competitors in the interaction between CK2 and [^{3}H]spermine.

Concentrations of analogs giving half-maximal displacement were calculated according to the method described by Job et al. (1978) and are represented in Figure 2.

Interestingly, spermine was identified as the best competitor of the CK2−[^{3}H]spermine interaction. Norspermine was as efficient as spermine and more active than bis(ethyl)-spermine and bis(ethyl)norspermine. The less charged polyamines spermidine and 1,12-diaminododecane as well as the longer analog Be3-8-3 were weaker compounds in displacing [^{3}H]spermine bound to CK2. Be4-4-4 was shown to be a less efficent competitor than 1,12-diaminododecane, spermidine, and Be3-8-3. Putrescine was determined to be the weakest ligand. Thus, the binding efficiency of different polyamine analogs used in this study could be ranked as follows: spermine and norspermine > bis(ethyl)spermine > bis(ethyl)norspermine > 1,12-diaminododecane, spermidine, and Be3-8-3 > Be4-4-4 > putrescine.

Comparison of the spermine analogs for their orders of activation and binding efficiencies toward CK2 showed them

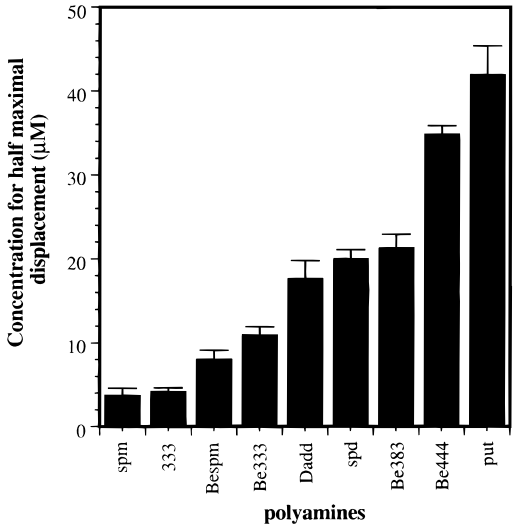

FIGURE 2: Interaction of polyamine analogs with CK2. Aliquots of CK2 (71 nM) were incubated with 0.25 μM [^{3}H]spermine for 5 min at 4 °C in the absence or presence of 20 μM spm, 3-3-3, Bespm, Be3-3-3, Dadd, spd, Be3-8-3, Be4-4-4, and put. Binding analyses were performed according to the rapid gel filtration centrifugation method described in Experimental Procedures. Concentrations of polyamine giving half-maximal displacement of [^{3}H]spermine were calculated according to the method described by Job et al. (1978).

to have a striking similarity; polyamines exhibiting the highest affinity for CK2 are the best CK2 activators. However, A_{50} values of all analogs were shown to be widely larger than the corresponding concentrations for half-maximal displacement of [^{3}H]spermine bound to CK2. Such a feature was already observed for spermine and was attributed to nonspecific interactions between polycationic compounds and casein (Moreno et al., 1993).

Analysis of the pH Effect on the Stimulation of CK2 Activity by Spermine. Polyamines exhibit primary and secondary amino groups whose p*K* values are in the range 8.2−11.0 (Hirschman et al., 1967). All the studies concerning the spermine−CK2 interaction have been performed in 10 mM Tris-HCl buffer at pH 7.4. Under these conditions, formation of a ligand−enzyme complex was driven essentially with a positively charged spermine molecule. Considering the possible ionic interactions between spermine and CK2, there should be an opportunity to decrease the binding efficiency and consequently the CK2 activation in modulating the pH value. Such a modulation should lead to the neutralization of the acidic amino acid residues in acidic conditions and of the ammonium spermine groups in alkaline conditions. With the aim of confirming the ionic feature of the spermine−CK2 linkage on one hand and as a guide for defining the involved amino acid residues of the β-subunit on the other hand, the stimulating effect of spermine on CK2 activity was analyzed toward casein in acidic conditions (pH 4.4 and 6.0) as well as in neutral (pH 7.4) and alkaline conditions (pH 9.0 and 10.4).

As shown in Figure 3, phosphorylation of casein by CK2 was well stimulated in the presence of submillimolar concentrations of spermine at pH 7.4.

The strongest CK2 activation was observed at a 500 μM spermine concentration, leading to an 8-fold stimulation of the kinase activity. Stimulation of CK2 activity by spermine was still detectable at pH 6.0 and 9.0. However, in these

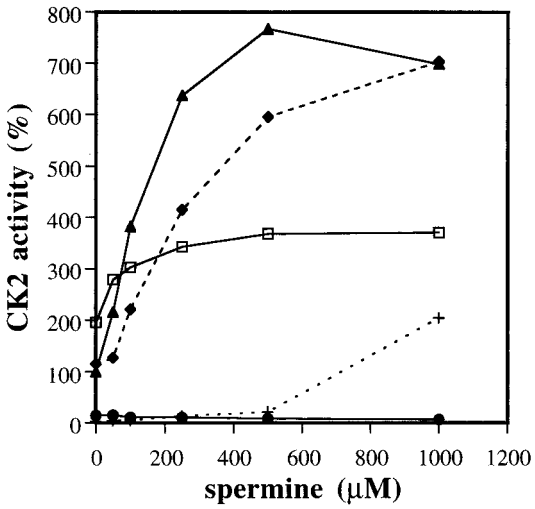

FIGURE 3: Effect of pH on the stimulation of CK2 activity by spermine. Aliquots of CK2 (3.6 nM) were incubated with 0.4 mg/mL casein, [γ-^{32}P]ATP (10^6 cpm/assay), 10 μM ATP, and 1 mM $MgCl_2$ at 30 °C for 8−15 min in the absence or presence of increasing spermine concentrations (50, 100, 250, 500, and 1000 μM) in the following pH conditions: 4.4 (●), 6.0 (□), 7.4 (▲), 9.0 (◆), and 10.4 (+). CK2 activity was calculated as the number of ^{32}P picomoles incorporated per minute and represented as the percent of the CK2 activity detected in the absence of polyamine at pH 7.4. The data are the results of three independent experiments.

conditions, the maximal stimulation was found to be close to 2 and 6, respectively. By contrast, the stimulation of CK2 activity in the presence of up to 500 μM spermine is completely prevented or strongly diminished at the extreme pH values of 4.4 and 10.4, respectively. However, in these conditions, the remaining CK2 activity is at least 5 times higher than the blank values (data not shown).

Taken together, these results suggest that the effect of spermine on CK2 activity is highly dependent on the pH. Neutralizing the spermine positive charges or the amino acid negative charges of the remaining active CK2 may result in a strong decrease in the spermine−CK2 interaction efficiency. This pH sensitive effect of spermine on CK2 activity provides evidence for the direct implication of glutamic or aspartic acids, whose p*K* values are close to 4, in the binding of spermine by CK2.

Characterization of the Interaction Number between Spermine and the CK2 Polyamine Binding Domain. The spermine molecule bears four positive charges at physiological pH (Hirschman et al., 1967). Thus, it would be crucial to determine how many of these charges are effectively involved in the spermine−CK2 complex. To answer this question, we have adopted the strategy used by Mascotti and Lohman (1990) for the study of DNA−polylysine complexes. In a first set of experiments, it was found that the spermine−CK2 interaction was highly salt-dependent, which was similar to their findings (data not shown). This indicated an electrostatic contribution to the binding reaction. Following the determination of the polyamine binding site at the primary structure level of the CK2 β-subunit (Leroy et al., 1995), we have overexpressed and purified a fusion protein involving the maltose binding protein and the β-domain encompassing residues Asp51−Pro110 (MBPβ51−110). Unlike the tetrameric form of CK2, the fusion protein exhibited a single high-affinity binding system for spermine (manuscript in preparation). This feature prompted us to use this fused domain in the following spermine binding experiments. Affinity constants of the binding between spermine and the fusion protein were determined at four distinct NaCl concentrations ranging from 25 to 75 mM (Figure 4). As shown in Table 3, raising the NaCl concentration from 25 to 75 mM leads to a decrease in the binding of spermine as indicated by the half-binding capacity values of 0.5, 1.0, 2.5, and 7.0 μM, respectively. K_{obs} values were obtained after a Scatchard plot analysis of the spermine binding data. According to Record et al., the dramatic decrease in K_{obs} observed with increasing Na^+ concentrations results from the increase in entropy upon release of counterions from the β-subunit into a solution of low salt concentration (Record et al., 1976a,b). The spermine dissociation constants are highly dependent on salt concentrations since they vary from 0.5 to 9.5 μM as the NaCl concentration is increased from 25 to 75 mM (Table 3). Thus, it clearly appears that the ionic strength modulates the CK2−spermine interaction, and as a consequence, this effect provides additional evidence for the involvement of ionic interactions. A regression plot

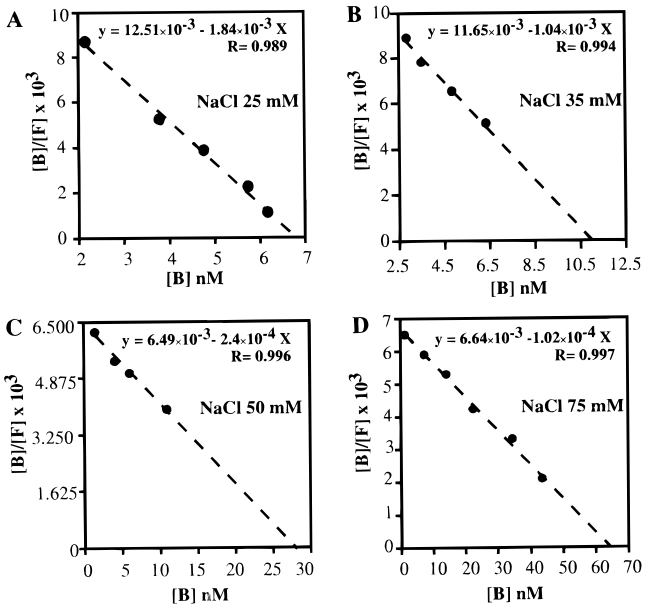

FIGURE 4: Analysis of the efficiency of the spermine−CK2 interaction as a function of the sodium chloride concentration. The fusion protein MBPβ51−110 was incubated with 0.25 μM [^{3}H]-spermine in the absence or presence of increasing concentrations of spermine. Binding analyses were performed by the rapid gel fitration centrifugation method described in Experimental Procedures for four concentrations of sodium chloride. Data were plotted according to Scatchard to determine the dissociation: (A) 25 mM NaCl, (B) 35 mM NaCl, (C) 50 mM NaCl, and (D) 75 mM NaCl. The data are the result of three independant experiments.

Table 3: Interaction Parameters of the Spermine-MBPβ51-110 Complex[a]

NaCl concentrations (mM)	25	35	50	75
half-binding capacities (μM)	0.5	1.0	2.5	7.0
dissociation constants (μM)	0.5	1.0	4.4	9.5

[a] Aliquots of MBPβ51-110 fusion protein (12.5 nM) were incubated for 5 min at 4 °C with 0.25 μM [^{3}H]spermine and increasing concentrations of unlabeled spermine. Binding analyses were performed in thepresence of 25, 35, 50, and 75 mM NaCl by the use of the rapid gel filtration centrifugation method described in Experimental Procedures. Half-binding capacities were determined for the four salt concentrations. Data were plotted according to Scatchard to determine the dissociation constant values of the spermine−β-domain complex for the four salt concentration conditions.

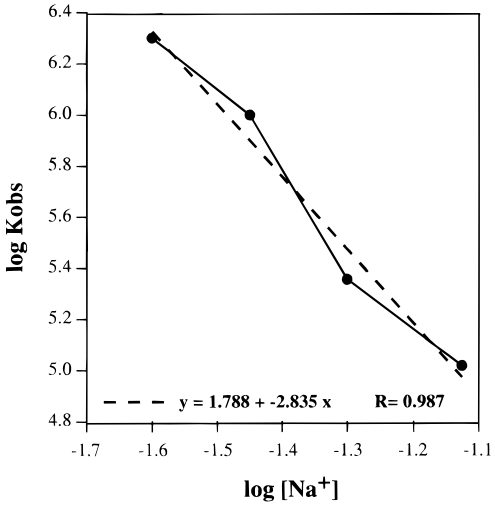

FIGURE 5: Representation of the counterion effect proceeding in the spermine−β complex. Data corresponding to log K_{obs} were plotted against log[Na^+] with the aim of verifying the equation log K_{obs}/log[Na^+] = $-\Psi z$, where K_{obs}, [Na^+], Ψ, and z are equivalent to $1/K_d$, the molar sodium concentration in the assay, the thermodynamic extent of counterion binding, and the ionic interactions number, respectively. The determination of Ψ was performed by analogy with the Ψ value used for the study of polycation−DNA interactions and must be considered therefore an approximation. The minus squares line was deducted from the obtained data.

of log K_{obs} as a function of log[Na^+] produces a slope which may be taken as $-\Psi z$ (Figure 5). The resulting z parameter deduced from the ratio slope/$-\Psi$ (where $\Psi = 0.79$ as set in Experimental Procedures) is determined to be 3.59. This value is in good agreement with the implication of the four positive charges of the spermine molecule in the formation of the spermine−β-subunit complex. Binding of a spermine molecule should therefore involve four ionic interactions with four amino acid residues of the CK2 β-subunit.

DISCUSSION

In this study, we explored the structural parameters of the polyamine−CK2 interaction. The use of different polyamine analogs in binding competition experiments as well as in CK2 activity assays has clearly shown that the polyamine molecules which exhibit the highest affinity for CK2 correspond to the best CK2 activators. A close examination of the polyamine analogs (Figure 6) has shown that molecules carrying less positive charges than spermine or whose aliphatic chains are longer than those of spermine are less efficient for both binding to CK2 and stimulation of CK2 activity. The scheme in Figure 6 takes into account the ionic interactions between the positive charges of the polyamine analogs and the negative charges of the β acidic residues. According to this model, the position of the positive charges along the aliphatic chain represents a crucial determinant for generating a tight interaction with the polyamine binding site of the β-subunit. The small increase in the aliphatic chain size found in the bis(ethyl)spermine molecule was not sufficient to strongly decrease the CK2 binding and the CK2 activation efficiency of this analog. The efficiency of norspermine and bis(ethyl)norspermine was found to be rather similar to the spermine one. Nevertheless, these two spermine analogs differ structurally from spermine in their central aliphatic chain which lacks a methylene group, and bis(ethyl)norspermine is longer than spermine. Furthermore, norspermine and bis(ethyl)norspermine do not exhibit any central symmetry as is the case for the spermine molecule, and as a result, the four positive charges reside on the same side of the longitudinal axis of these two molecules. The similarity in the observed effects of norspermine and spermine on CK2 is strong evidence suggesting that the structural differences between these two polyamines do not represent a crucial parameter changing drastically their interactions with the enzyme. The largest polyamines tested, Be3-8-3 and Be4-4-4, are poorly active on CK2 activity and are shown to be weak competitors of the binding of spermine. Three positive charges of spermine and Be3-8-3 are located in a position which allows these analogs to interact to some extent with CK2. However, spermidine which is shorter was found to be more efficient than Be3-8-3 in the stimulation of CK2 activity. Putrescine was found to be a very weak stimulator of CK2 activity and the weakest ligand among all analyzed. Comparison of the effects of spermine, spermidine, and putrescine on CK2 leads to the order spermine > spermidine > putrescine.

To obtain some further information on the nature of the amino acid residues of the CK2 β-subunit which are involved in the interaction, the effect of spermine on CK2 activity was analyzed in varying the pH values in the assay. Under these conditions, it was observed that the spermine effect on CK2 activity was highly pH-dependent since it was strongly attenuated in the extreme pH conditions of 4.4 and 10.4. In fact, the absence of stimulation of CK2 activity at pH 4.4 may reflect the neutralization of amino acid negative charges on the CK2 β-subunit or may be due to drastic modifications of the kinase structure upon pH variations. However, values for CK2 activity recorded at the extreme pH were at least 5 times higher than the blank values of the assay. These results suggest that extreme pH did not induce changes in CK2 conformation which could result in an irreversible loss of catalytic activity. The fact that, under these conditions, the effect of spermine on the remaining active kinase was abolished supports the notion of a role for the pH on the ionic state of both spermine and the CK2 polyamine binding site. However, slight changes in the conformation of the polyamine binding domain cannot be ruled out. Interestingly, in view of their pK values being close to 4.0, glutamic acid and aspartic acid represent good candidates for involvement in the spermine binding site of the β-subunit. Moreover, this is consistent with our previous identification of a spermine binding domain located in an acidic N-terminal cluster of 60 amino acid residues in the CK2 β-subunit (Leroy et al., 1995). In this study, a photoaffinity labeling method using a spermine analog allowed us to predict that glutamic residues 73 and 77 in this domain may participate in the electrostatic interactions with two of the four positive charges of the spermine molecule. In addition, mutant forms of the CK2 β-subunit in which glutamic acid residues 60, 61, and 63 have been replaced by alanine residues were poorly activated by polyamines (D. Leroy and J.-K. Heriché, unpublished results), in agreement with the observations of Boldyreff et al. (1993, 1994). In Figure 6, we show that four glutamic residues belonging to α-helices and separated by three amino acid residues may define the spermine binding site.

Evidence for the involvement of charged amino acid residues in the polyamine binding site of CK2 incited us to study the spermine binding affinity of the enzyme as a function of the ionic strength. The dissociation constant of

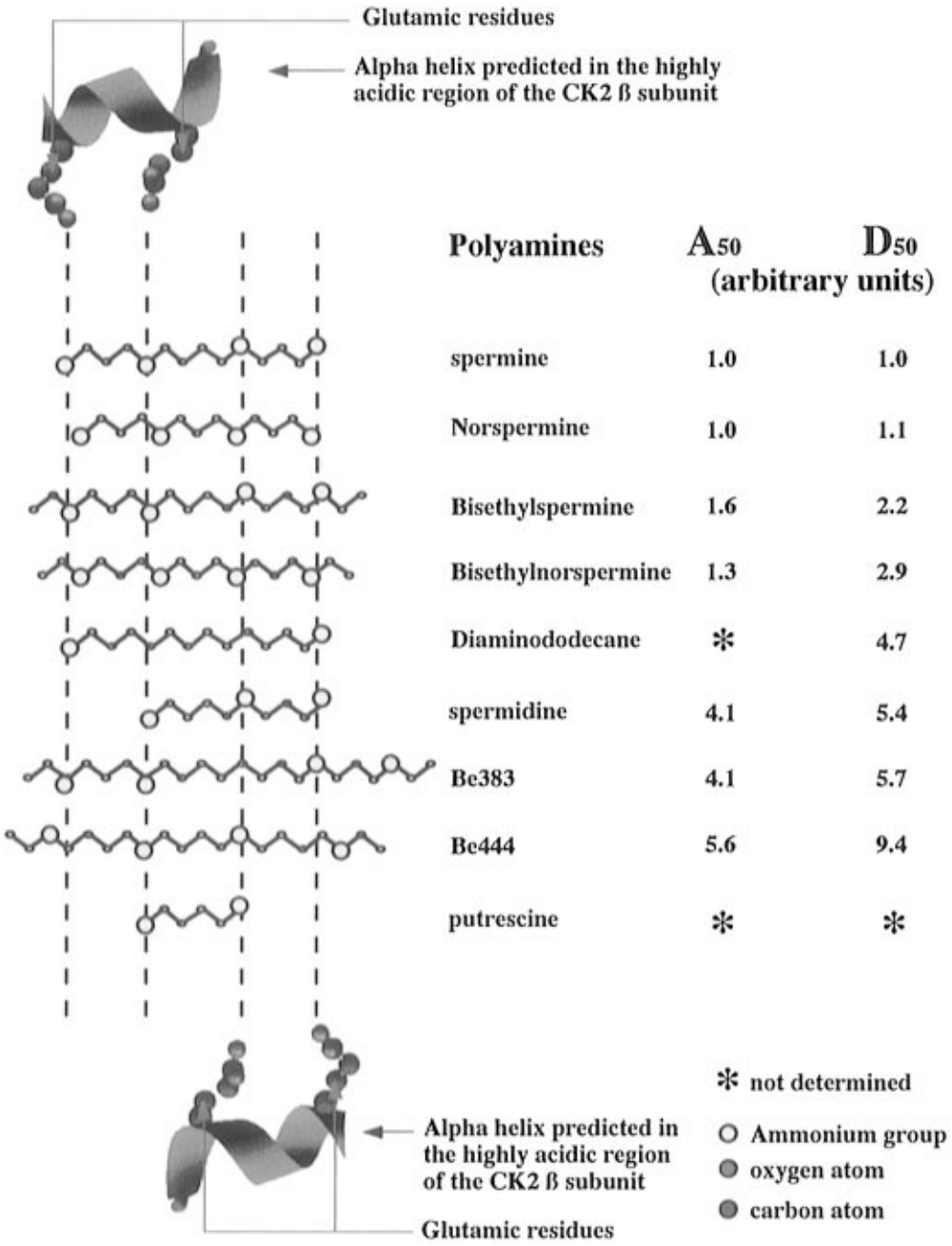

FIGURE 6: Schematic representation of the polyamine−CK2 interaction mechanism. The acidic amino acid residues involved in the CK2 polyamine binding site are represented on a ribbon helix. The methyl groups of the polyamines are represented by open circles and the positive ammonium groups by yellow circles. The spermine positive charges are drawn in a manner that allows optimal electrostatic interactions with the negative charges of the CK2 β-subunit. The spermine analogs are disposed with the aim of generating the highest number of electrostatic interactions with the negative charges of the spermine binding site. A_{50} and D_{50} values of polyamines (Table 2, Figure 2) were divided by the corresponding values recorded for spermine as a reference to allow comparison of the evolution of these parameters as a function of analog structure.

the spermine−CK2 complex was found to increase dramatically with the sodium chloride concentration. As suggested by Mascotti and Lohman (1991), such a phenomenon may be interpreted as the increase in entropy upon release of counterions from the β-subunit into a solution with a low salt concentration. Thus, it appeared that the ionic strength represents an important parameter controlling the CK2−polyamine interaction, and as a consequence, this effect provides additional evidence for the implication of ionic interactions in this complex.

Application of the equation $\log K_{obs}/\log[Na^+] = -\Psi z$ allowed us to predict that 4 ionic interactions are involved in the spermine−β subunit complex. The value of 3.5 determined by this way should be considered an approximate value because of the calculation mode of the constant z ($z = e^2/\epsilon KTb$). We decided to keep unchanged the absolute temperature T and the bulk dielectic constant ϵ in order to work with a general constant value represented by $e^2/\epsilon KT$. Such an approximation was justified by the temperature and the nature of the buffer used in the binding assay which are very similar to the experimental conditions previously used for the characterization of the polycation−DNA interaction. Moreover, the finding of 3.5 interactions in the formation of the spermine−β-subunit complex fits very well with the number of 3.7 previously determined using the Arrhenius law (Leroy et al., 1994).

Several studies have described structural modifications of DNA following the binding of polyamines. Some experiments led to results which are similar to the ones we obtained. For instance, Thomas and Thomas (1993) have established the order spermine > spermidine > putrescine on the basis of the efficiency of these polyamines in stabilizing triplex forms of DNA. In their study, the ionic charges are crucial for the interactions between polyamines and DNA. Similarly, Tassani et al. (1995) have shown that the three natural polyamines interact with mitochondria with the same order for their binding efficiencies.

A structural study using a photoactivatable analog of spermine allowed the elaboration of a model in which the polyamine molecule binds in the minor groove of DNA (Schmid & Behr, 1991). The polyamine is therefore able to crawl along the DNA molecule by the way of hydrogen bonds established between the ammonium groups of the polycation and the O2 atom of the nucleic base (Schmid & Behr, 1991). In such a case, the efficiency of the interaction would depend on the ammonium group number in the polyamine, and the largest polybasic molecule would be the best DNA ligand. Along the minor groove, no steric constraint would limit the binding of a polyamine, whatever its length. The positioning of the ammonium groups in the polyamine molecule probably represents an additional crucial parameter for the efficiency of the interaction with DNA. The remaining hydrogen bonds should be supported by a great similitude between the distances separating two consecutive positive ammonium groups of the polyamine and two consecutive base pairs. Thus, minor changes in the aliphatic central chain of spermine induces more effects on the interaction of polyamines with DNA than with CK2. In contrast, a length increase of the polyamine molecule by addition of aminopropyl groups should be deleterious for its binding to CK2 but could be a strong advantage for its interaction with DNA.

The three-dimensional structure of the polyamine binding site of the CK2 β-subunit can only accommodate the polyamine molecules which are able to minimize the steric interactions with the amino acid residues involved in the binding. From this view, one may consider the polyamine binding site as a cavity or a groove with an accurate topography. Thus, the polyamine binding site would be structurally defined to prevent the binding of polyamines larger than spermine. Minor changes in the central chain of spermine could be tolerated by the degree of freedom associated with the lateral chains of the acidic amino acid residues of the β-subunit involved in the interaction.

Our data provide strong evidence for the involvement of four ionic interactions between the positive charges of the spermine molecule and four acidic amino acid residues of the CK2 β-subunit. A fusion protein containing the β-domain (Asp51−Pro110) retains a high spermine binding activity, and the conformation of the polyamine binding site seems to be well-defined to allow only the binding of molecules whose structures are very similar to the one of spermine. It is hoped that these results will pave the way for the understanding of the spermine effect on CK2 activity at the molecular level. Obviously, elucidation of the three-dimensional structure of the β-subunit of CK2 is urgently required to shed some light on the involved mechanism.

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