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BIOCHEMISTRY

The effect of solvent composition on complex formation of S100B protein with peptides

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Abstract. Complex formation of a vertebrate calcium sensor protein S100B with peptides TRTKIDWNKILS (A) and SHLKSKKGQSTSRHKKLMWKTE (B) was studied. In contrast with previous reports, the affinity of these peptides for S100B was found to be similar. Added DTT, $CaCl_2$, NaCl, and change in pH differentially reduce the affinity of the two peptides up to two orders of magnitude in the studied range. The effect of added salt is more pronounced for binding peptide B with a higher positive net charge. We conclude that it is necessary to have carefully controlled media conditions in affinity measurements of S100B protein with its targets. This is important in search for efficient S100B blockers of medical interest.

Key words: bioorganic chemistry, S100B, protein, calcium sensor, peptide, charges, solvent.

INTRODUCTION

S100B protein is a Ca^{2+}-binding protein which was originally discovered in glial cells (Moore, 1965) and is now known to be differentially expressed in a wide variety of tissues and cell lines including malignant tumors. It belongs to the S100 subfamily of small calmodulin-like calcium sensors (Donato, 2003). The name S100 was given because these proteins are soluble in 100% saturated ammonium sulphate solution (Moore, 1965). More than twenty S100 proteins are known, with molecular masses 9–14 KDa.

S100B is one of the best studied members of the S100 family and has regulatory activities both as an intracellular and an extracellular protein. In cells, S100B is involved in signal transduction and Ca^{2+} homeostasis and participates in the regulation of cellular morphology. Extracellular S100B acts as a beneficial factor by stimulating neurite outgrowth and cell survival through interactions with the receptor for advanced glycan end products, when present at nanomolar concentration (Businaro et al., 2006). At micromolar concentration S100B stimulates the expression of pro-inflammatory cytokines and induces apoptosis (Donato, 2003).

S100B protein has attracted much attention over the past years, because in humans increased S100B levels have been detected with various clinical conditions: cancer, brain trauma, and ischemia; inflammatory, neurodegenerative, and psychiatric diseases. Overexpression of S100B in the brains of patients with Down's syndrome, Alzheimer's disease, and AIDS has led to the hypothesis that S100B plays a causal role in common neuropathologies associated with these diseases (Scotto et al., 1998).

Increased amounts of S100B are detected in tumours, e.g. in malignant melanoma (Harpio and Einarsson, 2004), and the S100B concentration correlates with the severity of the disease. When S100B binds to p53 protein, it inhibits the calcium-dependent phosphorylation of p53 by protein kinase C and prevents the formation of functionally active p53 tetramers. This may reduce the tumor suppressor function of p53, resulting in uncontrolled tumor growth. Down-regulation of S100B is a means to restore p53 levels (Lin et al., 2004). After cardiac surgery, cardiac arrest, stroke, or head injury S100B leakage into the extracellular fluids increases to toxic levels (Van Eldik and Wainwright, 2003). Measurements of the concentration of

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S100B are used in diagnostics to predict brain damage in cardiac surgery (Rothermundt et al., 2003).

S100B is a typical calcium sensor. It binds two Ca^{2+} ions per monomer and exposes hydrophobic surfaces that interact with target proteins (Ikura and Ames, 2006). It contains an abundance of negatively and positively charged surface residues located near the target binding area and the three-dimensional structure of the S100B–p53 peptide complex reveals several favourable hydrophobic and electrostatic interactions between S100B and the peptide in the binding pocket (Rustandi et al., 2000). S100B has been implicated in the calcium-dependent binding of at least 20 proteins in mammals (Donato, 2003). Binding areas in target proteins are usually amino acid sequences about 20 residues long. These sequences rarely contain negatively charged amino acids but are rich in basic residues and carry a net charge up to +8. An S100B consensus binding sequence [(R/K)(L/I)(XWXXIL)] was identified using a random bacteriophage library (Ivanenkov et al., 1995). A synthetic 12-residue peptide, TRTKIDWNKILS, derived from this sequence has been shown to interact with S100B in a calcium-dependent manner (Barber et al., 1999). This peptide has been reported to possess the highest known affinity for S100B (McClintock et al., 2002) and shown to compete for Ca^{2+}-S100B binding with other proteins including CapZ-α (Ivanenkov et al., 1995). It is remarkable that the peptide has a net charge of only +2.

Much weaker binding has been observed for a S100B-binding sequence from p53 protein (residues 367–388), SHLKSKKGQSTSRHKKLMPKTE, having a net charge of +8 (Rustandi et al., 1998). However, the medium conditions of affinity measurements for these two peptides were different with respect to pH, Ca^{2+}, and dithiotreitol (DTT) content. In the present paper we investigate how these media factors affect the complex formation between S100B protein and the two peptides chosen because of their different net charges.

MATERIALS AND METHODS

Chemicals

The purified S100B protein was a gift from S. Linse, produced in *Eschericia coli* BL21 De3 PLysS star using the plasmid with the rat S100B gene (kindly donated by D. Weber). Peptide A, TRTKIDWNKILS, was synthesized by standard solid phase methodology employing an Applied Biosystems model 431A Peptide Synthesizer. PAM resin and small-scale BOC 0.1 mM chemistry was used and the peptide was cleaved by the TFMSA method. Peptide B, SHLKSKKGQSTSRHKKLMWKTE, was a kind gift from S. Linse. It corresponds to residues 367–388 in the C-terminal regulatory domain of p53 protein, except that Phe385 is replaced by Trp. Purity of peptides was confirmed using reversed-phase C18 HPLC and mass spectrometry. Dithiotreitol, bis[2-hydroxyethyl] iminotris[hydroxymethyl]methane (bis-tris buffer, pKa = 6.5 at 25 °C, useful pH range 5.8–7.2), inorganic salts, and other reagents were purchased from commercial suppliers and used without further purification. The peptide concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of $5500\ M^{-1}\ cm^{-1}$ (Pace et al., 1995) for both tryptophane containing peptides and confirmed by amino acid analysis after acid hydrolysis. The concentration of S100B was determined by absorbance at 280 nm using an extinction coefficient of $2084\ M^{-1}\ cm^{-1}$, estimated by amino acid analysis after acid hydrolysis.

Fluorescence spectroscopy

Binding constants were measured in 10 mM buffer with 5 or 10 mM $CaCl_2$ at pH values 6 and 7. Dithiotreitol, NaCl, and extra $CaCl_2$ were added as required. The peptide concentration was between 0.3 and 2 μM, and S100B aliquots were added from a concentrated stock solution. Fluorescence emission spectra were obtained on a Perkin Elmer Luminescence Spectrometer LS 55 connected to the autopole system consisting of Julabo F25HD water bath set at 25 °C. Emission spectra were recorded between 310 and 400 nm using an excitation wavelength of 295 nm. Excitation and emission slits were set to 3–5 nm and 5–10 nm, respectively. Fifty scans were made after each added aliquot of S100B and spectra averaged. Titration curves for S100B–peptide complex formation were obtained by adding 15–20 aliquots of S100B up to a 3-fold excess of a protein over the peptide. This proved to provide a good data set for reliable calculation of $\log K$ values according to equation 2. Three to six independent titrations were performed at the same solvent composition using freshly made stock solutions. The $\log K$ values from parallel measurements were found within ±0.2 $\log K$ units of the calculated average $\log K$.

There is a small linear increase in fluorescence intensity with a maximum at about 340 nm when S100B aliquots, up to 2 μM total protein, are added to a solvent not containing peptide. We fitted protein–peptide titration curves also with equation 2, modified by adding a term linear to protein concentration. This did not result in a different binding constant value.

Examples of titration curves are shown in Fig. 1.

Data analysis

Data were analysed according to a 1 : 1 binding model

$$\mathrm{S100B} + \mathrm{pep} \Leftrightarrow \mathrm{S100B} \cdot \mathrm{pep}. \quad (1)$$

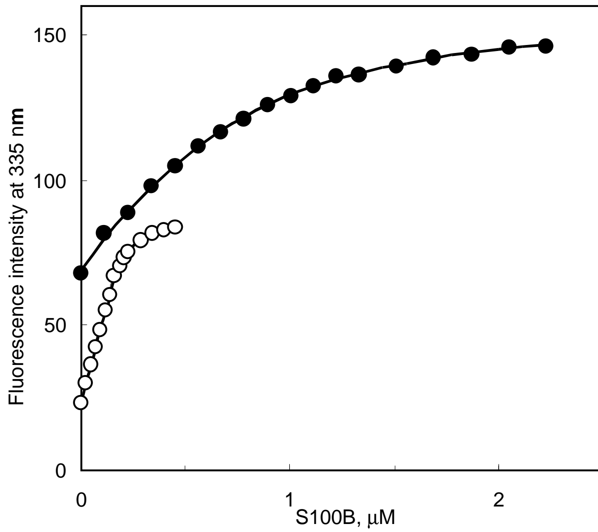

Fig. 1. Titration curves of S100B–peptide complex formation, monitored by changes in fluorescence intensity at 335 nm. Solid lines represent fitted curves according to equation 2. Filled symbols: 0.60 μM peptide A in 1 mM DTT, 40 mM $CaCl_2$, pH 7, 25 °C; Empty symbols: 0.20 μM peptide B in 5 mM $CaCl_2$, pH 7, 25 °C.

The concentration of free peptide after each addition can be calculated from the equation

$$C_{\rm pep}^{\rm free} = -\frac{1}{2}(C_{\rm pep}^{\rm tot} + K^{-1} - C_{\rm protein}^{\rm tot}) + \sqrt{\frac{1}{4}(C_{\rm pep}^{\rm tot} + K^{-1} - C_{\rm protein}^{\rm tot}K^{-1})^2 + C_{\rm protein}^{\rm tot}K^{-1}}, \quad (2)$$

where $C_{\rm pep}^{\rm free}$ is the free peptide concentration, $C_{\rm protein}^{\rm tot}$ the total S100B concentration, $C_{\rm pep}^{\rm tot}$ the total peptide concentration, K the stoichiometric binding constant. The total intensity at each titration point, $I_{\rm calc}$, is a combination of the intensity of free peptide, $I_{\rm free}$, and the intensity of the S100B–peptide complex, $I_{\rm bound}$, weighted by their respective concentrations according to the formula

$$I_{\rm calc} = \left(I_{\rm free} + (I_{\rm bound} - I_{\rm free}) \frac{C_{\rm pep}^{\rm free}}{K^{-1} + C_{\rm pep}^{\rm free}} \right) \frac{C_{p,i}^{\rm tot}}{C_{p,0}}, \quad (3)$$

where $C_{p,i}^{\rm tot}$ is the total peptide concentration after each addition and the ratio $C_{p,i}^{\rm tot}/C_{p,0}$ takes care of dilution effects. The binding curves are fitted directly to the experimental quantity using least squares fitting with Caligator software (André and Linse, 2002). All parameters were allowed to adjust in the fit ($C_{\rm pep}^{\rm tot}$, K, $I_{\rm free}$, $I_{\rm calc}$).

RESULTS

We have studied the effect of solvent composition on complex formation of S100B protein with two peptides of different net charges. Both peptides contain tryptophane for monitoring the complex formation via changes in fluorescence intensity. Peptide A, TRTKIDWNKILS, consists of 12 amino acids and has a net charge of +2. It is synthesized on the basis of a consensus sequence established for S100B binding bacteriophages (Ivanenkov et al., 1995). Peptide B, SHLKSKKGQSTSRHKKLMWKTE, consists of 22 amino acids and has a net charge +6 to +8 depending on the protonation state of two histidines. The peptide is an analogue of the S100B binding sequence in the p53 protein with phenylalanine-385 replaced by tryptophane. This peptide sequence is more typical of the target areas in many S100-binding proteins, considering its length and the net charge.

In the titration experiment for complex formation the maximum wavelength of fluorescence emission changes from 355 nm for tryptophane containing peptides to 335 nm for the mixture with S100B excess. Intensity increases about 2-fold. Emission maxima remain the same at all solvent conditions used; intensities vary slightly. A similar change in fluorescence has been previously described as indicating that the tryptophane residue of the peptide binds to a hydrophobic domain present in S100B (Rustandi et al., 1998). Three-dimensional structures for complexes of S100B with peptides A and B have been determined (1mq1.pdb, McClintock and Shaw, 2003; 1df7.pdb, Rustandi et al., 2000). S100B titrations were performed with a calmodulin-specific tryptophane containing peptide from smooth muscle myosin light chain kinase, residues 796–815 (André et al., 2004). Aliquots of S100B up to 30 μM total protein were added to a 5 μM peptide. About 1.7-fold linear increase in fluorescence intensity at 355 nm was observed against protein concentration. The maximum remained at 355 nm, which is characteristic of a peptide. We conclude that no significant stoichiometric binding took place in this case.

The results of affinity measurements are given in Table 1. The results show that the S100B protein binds both peptides with similar affinity. The $\log K$ value of 7.2 was observed for binding both peptides to S100B in the presence of 10 mM bis-tris buffer, 10 mM $CaCl_2$ at pH 7 and 25 °C.

It is obvious from the data that the affinity depends on the solvent composition. The presence of DTT and NaCl, and increase in the concentration of $CaCl_2$ were found to reduce the binding constant of both peptides to S100B protein. The affinity of both peptides is also reduced by changing the pH from 7 to 6. The observed total change due to media factors in the studied range is

Table 1. The effect of NaCl, $CaCl_2$, DTT, and pH on the affinity of S100B protein for two peptides in 10 mM bis-tris at 25 °C. K values were calculated as the mean of n parallel experiments at the same solvent conditions and the standard error of the mean is calculated for $\log K$ values

(A) S100B + peptide A (TRTKIDWNKILS), 10 mM bis-tris, 25 °C

pH	DTT, mM	$CaCl_2$, mM	NaCl, mM	log *K*	*n*
7	0	10	0	7.19 ± 0.19	5
7	1	10	0	6.84 ± 0.18	5
7	1	40	0	6.43 ± 0.14	3
7	1	10	100	6.52 ± 0.21	4
6	1	10	0	6.27 ± 0.16	3
7.2	0	10	50	6.7*	

* McClintock et al. (2002).

(B) S100B + peptide B (SHLKSKKGQSTSRHKKLMWKTE), 10 mM bis-tris, 25 °C

pH	DTT, mM	$CaCl_2$, mM	NaCl, mM	log *K*	*n*
7	0	5	0	8.16 ± 0.22	6
7	0	10	0	7.20 ± 0.16	5
7	1	5	0	7.27 ± 0.15	3
7	1	5	100	6.13 ± 0.18	4
6	1	5	0	6.31 ± 0.17	3
7.6	2	6	6	5.2**	

** Rustandi et al. (1998).

two orders of magnitude for peptide B and one order of magnitude for peptide A.

Added NaCl causes reduction in the binding constant for both peptides and the change is more pronounced for peptide B possessing a larger positive net charge. The reduction of affinity is also observed in case of the increased concentration of $CaCl_2$.

DISCUSSION

Many calmodulin-like calcium sensors bind and modulate multiple different targets. These sensor proteins are negatively charged at physiological conditions, and the binding areas in target proteins are highly positively charged. It is widely agreed that opposite charges are important in molecular recognition and formation of these complexes. In a recent review Ikura and Ames (2006) have concluded that molecular recognition and binding capacity of target proteins by calcium sensors depends on different patterns of charge distribution and hydrophobicity on protein surfaces. However, the exact role of charges in terms of their contribution to the affinity is not clearly understood. The response in affinity to significant changes in charge numbers is often weak or nearly nonexistent. In some cases the effect of added salt has been found to reduce the affinity between the calcium sensor and the target peptide, qualitatively as expected on the basis of the Coulomb law (Martin and Bayley, 2002). Some data, however, indicate that the binding affinity of the oppositely charged calcium sensor calmodulin and the target peptide actually increases tenfold with adding NaCl (André et al., 2006). The reasons for such a different behaviour in response to charge modifications and added salt are not clear.

Here, in case of binding the two peptides to S100B, we observe a behaviour which is in qualitative agreement with the Coulomb law. Added salt reduces the affinity of complex formation. The decrease is relatively small for peptide A with the net charge of +2 and significantly larger for peptide B with a high positive net charge. It is important to note that both NaCl and $CaCl_2$ cause a decrease in affinity, which is a normal behaviour in view of electrostatic screening by salt. For investigators of calcium sensor proteins the reduction of affinity due to added calcium might be surprising, as the general knowledge is that excess calcium has to be used. This is because the affinity of calcium sensors for target proteins is strictly dependent on the presence of Ca^{2+} ions in their specific sites. In many cases the apo-proteins (without bound Ca^{2+}) bind the targets by several orders of magnitude weaker than holo-proteins or do not bind significantly at all. For this reason, an excess of Ca^{2+} ions is commonly used in order to ensure that the calcium sites are filled and the protein is ready for action. S100B binds calcium ions with an effective equilibrium constant in a millimolar range. For this reason, 5 or 10 mM $CaCl_2$ is normally used when complex formation with peptides is under investigation. However, the data in this study show that although it is necessary to have calcium sites on S100B occupied, excess calcium also significantly reduces the affinity for highly charged peptides. The reduction of affinity due to both NaCl and $CaCl_2$ is more pronounced for a peptide with a higher net charge. Thus, in addition to enabling the peptide binding capacity via filling the calcium sites on S100B, $CaCl_2$ behaves as a common electrolyte which reduces the affinity of complex formation between oppositely charged reactants.

Another set of data which manifests the contribution of electrostatic interactions in the S100B–peptide complex formation is the effect of pH change on affinity. The affinity decreases with decreasing pH from 7 to 6 for both peptides. The reason for this change can be reduction of the negative net charge on the calcium sensor, as observed for calcium binding by calbindin D9k (Kesvatera et al., 2001), which also belongs to the subfamily of S100 proteins. Similar pH-dependence was

also observed for calmodulin–peptide complex formation (André et al., 2004). Alternatively, the reduction of affinity in this pH range could be explained as a result of proton uptake by His85 and His90 near the peptide site of S100B with the estimated pK_a values of 6.70 and 7.13, respectively (McClintock et al., 2002).

The amount of 1–5 mM DTT is routinely used in experiments with S100B to prevent possible formation of disulphide bridges between subunits in protein homodimer. In the experiments of Zn binding to S100B, performed by Wilder et al. (2003), it was necessary to avoid DTT. The authors claimed not having seen any difference in the ability of S100B to bind calcium or target protein, nor to affect the aggregation state of the protein whether the solvent was present or absent. In contrast to this, we establish DTT as a strong agent in reducing the affinity of S100B for both peptides, the decrease being more pronounced with peptide B. The presence of 1 mM DTT reduces the affinity about tenfold, and another tenfold decrease becomes obvious from the earlier data, showing $\log K$ of 5.2 in the presence of 2 mM DTT for binding the p53-peptide to S100B (Rustandi et al., 1998). Therefore, DTT should be avoided or kept at minimum in order to increase the sensitivity of affinity measurements.

In low salt buffer and the absence of DTT S100B shows stronger affinity for peptide B with a higher positive net charge. Added inorganic salt, whether NaCl or $CaCl_2$, reduces the affinity of S100B for peptides, the decrease being clearly more pronounced in case of peptide B with a higher positive net charge. The affinity for both peptides is also reduced by the change in pH from 7 to 6. The change is greater for peptide B. As a result, at ionic strength values higher than 100 mM the affinity of peptide B becomes weaker than that for peptide A. This is in agreement with the earlier data, showing that peptide A is a more efficient binder to S100B than peptide B, in the presence of high salt (Rustandi et al., 1998).

CONCLUSIONS

The main purpose of this study was to see whether added salt affected complex formation, indicative of the presense of measurable electrostatic contribution to the affinity. We find that added salt reduces the affinity of S100B for peptides and the change is more pronounced in case of the peptide with a high positive net charge. We conclude that S100B–peptide complexes are suitable for further studies of salt-dependent ionic interactions in protein–peptide complexes. The acidity of the medium and DTT were also found to have a substantial effect on the affinity and need to be carefully controlled in measurements of S100B–peptide complex formation.

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Solvendi koostise mõju S100B-valgu kompleksi moodustumisele peptiididega

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On uuritud selgroogsetes organismides esineva kaltsiumi sensorvalgu S100B kompleksi moodustumist peptiididega TRTKIDWNKILS (A) ja SHLKSKKGQSTSRHKKLMWKTE (B). Erinevalt varasematest määrangutest on peptiidide afiinsus S100B suhtes osutunud samasuguseks. Keskkonda lisatud DTT, $CaCl_2$, NaCl ja pH muutmine uuritud vahemikus vähendavad peptiidide afiinsust S100B suhtes kuni kaks suurusjärku. Soolalisandi mõju sidumiskonstandile on suurem peptiid B korral, mille positiivne laeng on suurem. Tulemused näitavad, et mõõtes S100B afiinsusi eri partnerite suhtes on olulised rangelt kontrollitud keskkonnatingimused. See on tähtis meditsiinilist huvi pakkuvate S100B-blokaatorite otsingul.