Conformational and thermodynamic properties of peptide binding to the human S100P protein

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Abstract

S100P is a member of the S100 subfamily of calcium-binding proteins that are believed to be associated with various diseases, and in particular deregulation of S100P expression has been documented for prostate and breast cancer. Previously, we characterized the effects of metal binding on the conformational properties of S100P and proposed that S100P could function as a Ca^{2+} conformational switch. In this study we used fluorescence and CD spectroscopies and isothermal titration calorimetry to characterize the target-recognition properties of S100P using a model peptide, melittin. Based on these experimental data we show that S100P and melittin can interact in a Ca^{2+} -dependent and -independent manner. Ca^{2+} -independent binding occurs with low affinity ($K_d \approx 0.2$ mM), has a stoichiometry of four melittin molecules per S100P dimer and is presumably driven by favorable electrostatic interactions between the acidic protein and the basic peptide. In contrast, Ca^{2+} -dependent binding of melittin to S100P occurs with high affinity ($K_d \approx 5$ μM) has a stoichiometry of two molecules of melittin per S100P dimer, appears to have positive cooperativity, and is driven by hydrophobic interactions. Furthermore, Ca^{2+} -dependent S100P-melittin complex formation is accompanied by significant conformational changes: Melittin, otherwise unstructured in solution, adopts a helical conformation upon interaction with Ca^{2+} -S100P. These results support a model for the Ca^{2+} -dependent conformational switch in S100P for functional target recognition.

Keywords: Isothermal titration calorimetry; circular dichroism spectroscopy; fluorescence spectroscopy; structural thermodynamics; conformational transition

The human protein S100P belongs to the S100 subfamily of calcium-binding proteins that share a common Ca^{2+} -binding structural motif, the EF-hand (Kretsinger 1976; Chazin 1995). Twenty members of the subfamily have been identified to date (Donato 1999; Gribenko et al. 2001). These proteins have two EF-hand motifs in their primary structure,

various metal-binding properties, and are expressed in a tissue- and cell-type-specific fashion (Hilt and Kligman 1991; Zimmer et al. 1995; Schafer and Heizmann 1996). Increased or altered expression of S100 proteins has been documented for many human diseases and tumors (Donato 1999). S100P was shown to be differentially expressed in androgen-dependent and androgen-independent prostate cancer cell lines (Averboukh et al. 1996). Recently it has also been shown that S100P overexpression is associated with immortalization of human breast epithelial cells in vitro and early stages of breast cancer development in vivo (Guerreiro Da Silva et al. 2000).

In our previous report we characterized the oligomerization and divalent cation-binding properties of S100P and proposed a $\text{Ca}^{2+}/\text{Mg}^{2+}$ switch model for S100P function (Gribenko and Makhatazde 1998). According to this model,

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Abbreviations: ΔASA , change in water accessible surface area; ITC, isothermal titration calorimetry; CD, circular dichroism; $[\text{MEL}]_1$, concentration of melittin; $[\text{S100P}]_1$, concentration of S100P monomer; $[\text{S100P}]_2$, concentration of S100P dimer; apo-S100P, S100P without Ca^{2+} ; Ca^{2+} -S100P, Ca^{2+} -loaded S100P.

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S100P exists in solution as a homodimer, and each monomer has two metal-binding sites located in the C-terminal and N-terminal loops, with high and low affinity for calcium, respectively. The low affinity Ca^{2+} -binding site is also able to bind Mg^{2+} at physiological concentrations of this ion, and the binding of magnesium increases the affinity for Ca^{2+} at the high-affinity site. Metal binding is accompanied by conformational changes in S100P that result in the exposure of hydrophobic surfaces. These results indicate that Ca^{2+} -S100P has the potential to interact with other intracellular target proteins through hydrophobic interactions (Gribenko and Makhatazde 1998). To test this hypothesis, we have analyzed the binding of a model amphipathic peptide, melittin, to S100P. Melittin has been widely used to study protein-peptide interactions of other Ca^{2+} -binding proteins, such as calmodulin, and has proven to be a useful model (e.g., Comte et al. 1983; Scaloni et al. 1998; Weljie and Vogel 2000). Melittin is a linear peptide of 26 amino acid residues, with the sequence GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂. In solutions of low ionic strength and neutral pH and at low peptide concentrations (<100 μM), melittin exists as an unstructured monomer. In contrast, at high ionic strength, alkaline pH, and/or high (>0.5 mM) peptide concentrations, the equilibrium shifts toward a tetramer formed by amphipathic α -helices of melittin (Wilcox and Eisenberg 1992; Hagihara et al. 1994).

In this work, the interactions of S100P with melittin were analyzed by fluorescence and CD spectroscopies and ITC. S100P/melittin interactions that occur in the absence of Ca^{2+} have low affinity ($K_d \approx 200 \mu\text{M}$) and a stoichiometry of two molecules of melittin bound per monomer of S100P. The S100P/melittin complex formed in the presence of saturating Ca^{2+} concentrations is tight ($K_d \approx 5 \mu\text{M}$), has a stoichiometry of two molecules of melittin bound per S100P homodimer, and appears to have positive cooperativity ($\Delta\Delta G_{\text{coop}} = -4 \pm 1 \text{ kJ/mole}$). Binding of melittin to Ca^{2+} -S100P is driven by hydrophobic interactions, as indicated by the large negative heat-capacity changes upon complex formation. The complex formation is also accompanied by conformational changes, leading to a coil-helix transition in melittin. Based on these results we propose a model of how a Ca^{2+} -conformational switch in S100P might exert function through the interaction with cellular targets.

Results and Discussion

S100P-melittin interactions characterized by ITC

ITC is a method of choice for measuring the energetics of protein-ligand interactions. In ITC experiments the heat absorbed or released upon ligand binding to a macromolecule is measured (see Fig. 1 for a representative example of ITC experiment). The heat of binding is proportional to the enthalpy and degree of binding. Thus from ITC titrations at a

Fig. 1. Representative isothermal titration calorimetry experiment. Heat effects recorded as a function of time during 25 successive 7- μL injections of 1.18 mM solution of S100P into the cell containing 0.06 mM solution of melittin at 32°C. Buffer conditions were 50 mM Tris, 0.2 mM EDTA, 5mM CaCl_2 , 1.5 mM TCEP at pH 7.5.

single temperature it is possible to obtain the enthalpy of interactions (ΔH_o), as well as the affinity (K_d) and stoichiometry (N_b) of these interactions (Wiseman et al. 1989; Lopez and Makhatazde 2002).

Figure 2 shows the heat absorbed upon the titration of a melittin solution with S100P in the absence of Ca^{2+} as a

Fig. 2. The cumulative heat of melittin titration with apo-S100P as a function of S100P concentration (bottom X-axis) at different temperatures are shown by open symbols (circles, 12°C; triangles, 17°C; and squares, 32°C). The enthalpy of reverse titration of S100P with melittin as a function of melittin concentration (top X-axis) are shown by solid symbols (circles, 12°C; triangles, 17°C; and squares, 32°C). Solid lines are the fit of the experimental data to the equation 1 with the parameters shown in Table 1. Experimental data for 25°C were omitted for clarity. Vertical arrow shows the approximate concentration of melittin above which the population of monomers becomes >80%.

function of S100P concentration. The reaction was endothermic and never reached apparent saturation even at relatively high (up to 15:1) S100P to melittin ratios, suggesting an interaction with low affinity. To gain more insight into the binding reaction, the reverse titrations were performed at the same temperature. In reverse titrations, the S100P solution in the ITC cell was titrated with melittin. Melittin has a low affinity to apo-S100P; therefore, to measure a complete titration profile, the concentrations of melittin during the titration should cover a broad range. However, melittin has tendency to form tetramers at high concentrations (Terwilliger and Eisenberg 1982; Goto and Hagihara 1992; Wilcox and Eisenberg 1992). Thus we limited experimental conditions to the concentrations of melittin in which at least 80% of molecules are monomeric (i.e., [MEL] > 200 μ M. For the reverse titrations the heat absorbed per mole of S100P monomer appears to be about twofold larger than the heat absorbed per mole of melittin (Fig. 2). This indicates that two molecules of melittin bind per S100P monomer in the absence of Ca^{2+} . Using the stoichiometry, N_b , for the melittin interaction with S100P in the absence of Ca^{2+} , the other two parameters (K_d and ΔH_o) were determined from the fit of the titration curve to equation 1. Titrations were performed at different temperatures to calculate the temperature dependence of the enthalpy of binding, which is described by the heat-capacity changes upon complex formation, ΔC_p . The enthalpy of binding and K_d at each temperature obtained from the fit are summarized in Table 1. Two major conclusions can be drawn from these results. First, the interactions of apo-S100P with melittin have relatively low affinity ($K_d \approx 200 \mu\text{M}$ at 25°C; Table 1). Second, it seems that the enthalpy of binding of melittin to apo-S100P is independent of temperature, which means that the

heat-capacity change upon binding is close to zero (Fig. 3). An absence of the heat-capacity change upon melittin binding to apo-S100P implies that no hydrophobic surfaces are buried or exposed in the apo-S100P/melittin complex (Makhatadze and Privalov 1995). Alternatively, in addition to the changes in hydrophobic surface area upon S100P/melittin complex formation, there are also even larger (see equation 9) changes in the polar and charged surface area that will lead to small overall ΔC_p . It can be suggested that apo-S100P melittin interactions are mainly electrostatic. Indeed, S100P is an acidic protein, which will carry an overall negative charge at neutral pH. Melittin, on the other hand, is a basic peptide and will be positively charged at neutral pH. The usual experimental way of validating the electrostatic character of interactions is to measure the effect of ionic strength on the thermodynamics of complex formation. Unfortunately this approach cannot be used for the apo-S100P/melittin system because melittin oligomerizes at high ionic strength (Hagihara et al. 1992; Wilcox and Eisenberg 1992). Thus we cannot deduce the exact mechanism of the apo-S100P/melittin interactions and can only conclude that hydrophobic interactions do not play a dominant role for this complex formation.

Melittin titrations with S100P at different temperatures in the presence of 5 mM Ca^{2+} are shown in Figure 4. Three major differences are observed when comparing the titrations in the presence and absence of Ca^{2+} . First, the heat effect rapidly reaches saturation in the presence of Ca^{2+} , which indicates a much higher affinity of melittin to Ca^{2+} -S100P than to apo-S100P. Second, the binding curves reach saturation at a Ca^{2+} -S100P/melittin ratio of ~ 1 , indicating that only one molecule of melittin binds per monomer of Ca^{2+} -bound S100P. Third, the enthalpy of the S100P/melittin complex formation in the presence of Ca^{2+} is smaller

Table 1. Thermodynamic parameters for melittin binding to S100P in the absence and presence of Ca^{2+} at different temperatures obtained from ITC experiments

- Ca^{2+}			5 mM Ca^{2+}		
T ($^{\circ}\text{C}$)	N_b	K_d (μM)	ΔH_o (kJ/mole) ^a	T ($^{\circ}\text{C}$)	ΔH_o (kJ/mole) ^b
12		300 ± 100	62 ± 6	12	25 ± 4
17		270 ± 90	66 ± 6	17	15 ± 3
	2			1	<10
25		180 ± 60	63 ± 6	25	-5 ± 2
32		80 ± 30	57 ± 6	32	-21 ± 3

The thermodynamic parameters of S100P-melittin interactions were obtained from the simultaneous fit of all data. Uncertainty values for ΔH_o are largely defined by the $\sim 7\%$ error in concentration measurements.

^a The ΔH_o values reported are per mole of S100P, and because the stoichiometry of interactions is two molecules of melittin per S100P monomer, the enthalpy of binding of one molecule of melittin will be half that value, as shown in Fig. 3.

^b The ΔH_o values reported are per mole of either S100P or melittin because the stoichiometry of interactions is 1:1.

Fig. 3. Temperature dependence of the enthalpy of binding of one molecule of melittin to S100P in the absence (solid circles) and in the presence (open circles) of Ca^{2+} . Solid lines represent a linear fit of the data with the slope representing the heat capacity changes, ΔC_p , upon complex formation: ΔC_p in the absence of Ca^{2+} is $-0.1 \pm 0.5 \text{ kJ}/(\text{mole} \cdot \text{K})$; ΔC_p in the presence of Ca^{2+} is $-2.3 \pm 0.5 \text{ kJ}/(\text{mole} \cdot \text{K})$.

Fig. 4. The cumulative heat of melittin binding to Ca^{2+} -S100P as a function of S100P/melittin ratio at different temperatures: inverted solid triangles, 12°C; solid squares, 17°C; solid circles, 25°C, and solid triangles, 32°C. The vertical dashed line shows the 1:1 ratio of S100P to melittin.

than in the absence of Ca^{2+} and more importantly strongly depends on temperature. ΔH_o is endothermic at 12°C and 17°C and becomes exothermic at 25°C and 32°C (Fig. 4). This result indicates that the S100P/melittin complex formation in the presence of Ca^{2+} is accompanied by a negative heat-capacity change, defined by the slope of the ΔH_o dependence on temperature (Fig. 3). The binding described by the titration curves shown in Figure 4 is very close to stoichiometric. Under stoichiometric conditions ΔH_o and N_b can be readily calculated but reliable calculation of K_d is impossible. Nevertheless, we can estimate that K_d is $<10 \mu\text{M}$ (based on the concentrations of protein and ligand in these experiments), which is at least an order of magnitude tighter than melittin/S100P complex formation in the absence of Ca^{2+} .

The data shown in Figure 4 clearly indicates that the S100P/melittin complex formation in the presence of Ca^{2+} is accompanied by a large heat-capacity change, defined as the slope of ΔH_o as a function of temperature, $\Delta C_p = -2.3 \pm 0.5 \text{ kJ}/(\text{mole K})$. It is well accepted that the heat-capacity change for a protein–ligand interaction can also be estimated from the changes in water-accessible surface area (e.g., see Brox et al. 2001 and references therein). Because the three-dimensional structure of the S100P-melittin complex has yet to be solved, we produced a model to predict the complex structure. The changes in the accessible surface areas upon complex formation were estimated as described by equation 8 in Materials and Methods and they were used to calculate ΔC_p according to equation 9. The calculated ΔC_p $-1.7 \text{ kJ}/(\text{mole K})$ agrees reasonably well with the experimental value of $-2.3 \pm 0.5 \text{ kJ}/(\text{mole K})$, considering that we used a model as a structure. To further characterize the structural properties of melittin/S100P

complex in the presence and absence of Ca^{2+} , we used CD spectroscopy.

Analysis of S100P/melittin complex formation by far-UV CD spectroscopy

Far-UV CD spectroscopy is commonly used to monitor changes in secondary structure of proteins, and we applied this technique to investigate if binding of melittin to S100P is accompanied by any structural changes in the peptide. Both S100P and melittin have non-zero ellipticity in the far-UV CD spectra. Thus, to analyze the changes in the structure of melittin upon binding to S100P, we monitored the differences in the ellipticity ($\Delta\theta$) of melittin solutions in the presence of S100P and those of melittin solutions of equal concentration in the absence of S100P. Figure 5A shows the changes in $\Delta\theta$ at 222 nm as a function of the [melittin]/[S100P] ratio. In the presence of 5 mM Ca^{2+} , titration of S100P with melittin produces a sharp increase in $\Delta\theta_{222}$ until the ratio of [melittin]/[S100P] reaches 1. Further increases in melittin concentrations do not produce any significant changes in $\Delta\theta$. This indicates that under these experimental conditions, binding of melittin to Ca^{2+} -S100P is stoichiometric and the complex is formed at the ratio of one molecule of melittin bound per monomer of Ca^{2+} -S100P. In contrast, in the absence of Ca^{2+} , relative changes in far-UV ellipticity are much smaller and do not appear to reach saturation (i.e., binding is not stoichiometric; Fig. 5A). This result further supports the conclusion suggested by the ITC titration experiments (i.e., the affinity of melittin

Fig. 5. (A) Changes in the ellipticity at 222 nm at 20°C as a function of melittin to S100P ratio. Solid circles show changes in melittin ellipticity in the presence of apo-S100P (30 μM), as compared to the ellipticity of melittin alone at the same peptide concentration. Open circles show changes in melittin ellipticity in the presence of Ca^{2+} -S100P (30 μM), as compared to the ellipticity of melittin alone at the same peptide concentration. Changes in ellipticity at each point calculated as percentage of maximal decrease in ellipticity of melittin in the presence of Ca^{2+} -bound form of S100P. Vertical dotted line shows the 1:1 ratio of melittin to S100P. The dashed line is a simulated binding curve based on the parameters listed in Table 1. Solid lines are drawn to guide the eye. (B) Far-UV CD spectra of melittin when free in solution (solid triangles) and when in complex with Ca^{2+} -S100P (open circles).

Table 2. Fluorescence emission maxima ($\lambda_{\max}$) and Stern-Volmer constants (K_{sv}) of melittin under various conditions

		Melittin only		Melittin/S100P	
		$\lambda_{\max}$ (nm)	K_{sv} (M ⁻¹)	$\lambda_{\max}$ (nm)	K_{sv} (M ⁻¹)
Ca ²⁺	0	354 ± 0.5	11.4 ± 0.1	348 ± 0.5	6.5 ± 0.1
	5 mM	354 ± 0.5	12.1 ± 0.1	345 ± 0.5	4.6 ± 0.1

to apo-S100P is much lower than to Ca²⁺-S100P and that the stoichiometry of melittin:Ca²⁺-S100P interactions is 1:1).

The fully ligated Ca²⁺-S100P/melittin complex can be populated at reasonably low concentrations of melittin and thus makes it possible to evaluate structural changes that accompany complex formation. The spectrum of free melittin indicates that melittin does not have a well-defined secondary structure under the given experimental conditions (Fig. 5B). The difference between the spectra of the Ca²⁺-S100P/melittin complex and S100P alone shows that there is a large increase in the far-UV CD signal intensity (see Fig. 5A). This difference in the CD signal is due to the conformational changes that occur in S100P and/or melittin. Structural studies showed that peptide binding to S100A10 and S100B either does not induce any changes in secondary structure (Rety et al. 2000) or leads to the folding of just 5 residues at the C terminus (Rustandi et al. 2000) and thus should not contribute much to the far-UV CD signal. Thus the changes in the far-UV CD signal were attributed to the

structural rearrangement in the melittin molecule. To obtain the spectrum of melittin in complex with S100P, we subtracted the spectrum of the solution of Ca²⁺-S100P alone from the spectrum of the solution containing both Ca²⁺-S100P and melittin. Far-UV CD spectra of melittin in complex with Ca²⁺-S100P at a 1:1 molar ratio are presented in Figure 5B. The shape of the spectrum is typical of that of an α -helix with ellipticity minima at 222 and 208 nm. Furthermore, the ellipticity value of $-20,000 \text{ deg}\cdot\text{cm}^2\cdot\text{dmole}^{-1}$ at 222 nm is within the experimental error of the reported ellipticity values of $-22,000 \text{ deg}\cdot\text{cm}^2\cdot\text{dmole}^{-1}$ at 25°C for melittin in an α -helical conformation (Hagihara et al. 1994). From this we can conclude that the changes in the far-UV CD spectra result from the formation of a helical structure in melittin upon binding to the Ca²⁺-S100P. This finding is in agreement with the crystal and solution structures of other S100 proteins with their peptide targets that show a helical structure of the peptide in the complex (Rety et al. 1999, 2000; Rustandi et al. 2000). The CD experiments indicate that the binding of melittin to Ca²⁺-S100P is accompanied by the folding of this peptide, and the ITC experiments indicate that the melittin-Ca²⁺-S100P complex is stabilized by hydrophobic interactions. Therefore, the only Trp residue of melittin, Trp 19, might be involved in these interactions. To test this hypothesis we used fluorescence spectroscopy.

S100P-melittin interactions monitored by fluorescence spectroscopy

Fluorescence spectroscopy appears to be a useful tool to follow the S100P-melittin interactions. S100P does not have any tryptophan residues, but has two tyrosines (Tyr 18 and Tyr 88), which define a typical tyrosine fluorescence emission spectrum of S100P (Gribenko et al. 1998). Melittin has a single tryptophan residue (Trp 19). Thus, if melittin/S100P interactions involve this tryptophan, it is expected that complex formation will have an effect on the fluorescence intensity and/or fluorescence emission maximum of Trp.

The fluorescence emission maximum of tryptophan, $\lambda_{\max}$, in a polar environment, such as an aqueous solution, is $\sim 354\text{--}355 \text{ nm}$. In nonpolar environments, such as a protein interior, the maximum emission shifts to lower wavelengths (Lakowicz 1983). These changes in the emission maximum can be used to monitor relative exposure of tryptophans. Another method that is commonly used to estimate relative exposure of tryptophan residues is the quenching of the fluorescence signal by external quenchers, such as acrylamide, iodine, oxygen, and others (Eftink and Ghiron 1976; Lakowicz 1983). Quenching efficiency can be characterized by the Stern-Volmer constant (K_{sv}), which is defined as an inverse of the concentration of the quencher at which half of the fluorescence intensity of the fluorophore is quenched

Fig. 6. Relative changes in melittin fluorescence intensity upon titration with S100P. Filled circles show fluorescence intensity at each titration point when experiments are performed in the absence of Ca²⁺. Open circles show fluorescence intensity at each titration point when experiments are performed in the presence of 5 mM Ca²⁺. The dashed and dotted lines show the fit of experimental data to the two-independent-binding-site model described by the equation 7, with the binding constant 5.3 μM . The solid line shows the fit of the experimental data to the model with two cooperative binding sites described by the equation 6, with the macroscopic binding constants $K_1 = 5 \pm 2 \mu\text{M}$ and $K_2 = 6 \pm 2 \mu\text{M}$.

(Eftink and Ghiron 1976; Lakowicz 1983). A higher K_{sv} indicates that the fluorophore is more accessible to the quenching agent and therefore is more exposed. Thus changes in tryptophan fluorescence emission maximum and changes in K_{sv} should correlate if a process leads to changes in the environment of the tryptophan due to burial of the residue in the interior of the protein.

Table 2 presents λ_{max} and K_{sv} values for the titration of melittin with acrylamide in the presence or absence of S100P with or without Ca^{2+} . In the absence of S100P and Ca^{2+} , the maximum fluorescence emission wavelength of melittin is 354 nm, as expected for a tryptophan residue exposed to aqueous environment. Under the same conditions, the K_{sv} value is 11.4 M^{-1} (Table 2), again indicating that the tryptophan is solvent-exposed (Eftink and Ghiron 1976). Addition of 5 mM Ca^{2+} to the melittin solution does not change λ_{max} and has little effect on the K_{sv} values. These results suggest that addition of 5 mM Ca^{2+} does not significantly affect fluorescent properties of the tryptophan residue of melittin. Therefore any changes in λ_{max} or K_{sv} in the presence of S100P would result from the interaction of S100P and melittin. In the presence of apo-S100P, the fluorescence-emission maximum shifts to 348 nm, concomitant with a decrease in K_{sv} to 6.5 M^{-1} (Table 2). These results indicate that in the presence of apo-S100P, the tryptophan residue of melittin is less exposed to the aqueous environment, as compared to free melittin under similar conditions (Table 2). Because at low ionic strength and low peptide concentrations melittin is largely unstructured (Goto and Hagihara 1992; Hagihara et al. 1992; Wilcox and Eisenberg 1992), the effect is due to binding of melittin to apo-S100P. Titration of the solutions containing both S100P and melittin with acrylamide in the presence of 5 mM Ca^{2+} produces the lowest value of K_{sv} at 4.6 M^{-1} . The maximum in the emission spectrum of melittin in the presence of Ca^{2+} also has the lowest value, $\lambda_{max} = 345 \text{ nm}$. Thus Trp 19 of melittin is indeed involved in the formation of hydrophobic interactions on the binding interface of the melittin/ Ca^{2+} -S100P complex.

Figure 6 presents the changes in the fluorescence intensity at 353 nm after excitation at 295 nm upon titration of melittin with S100P. The relative fluorescence intensity of melittin, I/I_{max} , increases with the increase in Ca^{2+} -S100P concentrations. Furthermore, the shape of the profile in the presence of Ca^{2+} resembles an equilibrium titration curve with the saturation achieved at concentrations of S100P dimer close to $15 \mu\text{M}$. Addition of apo-S100P also leads to changes in I/I_{max} . However, these changes are much lower in magnitude and appear to be rather monotonic in the concentration range studied (up to 5:1 ratio of S100P/melittin). These results indicate that even if both the apo form and Ca^{2+} form of S100P can bind melittin, the binding affinity is much higher for Ca^{2+} -S100P in agreement with the CD and ITC results.

It is important to mention that fluorescence experiments are normally performed at much lower concentrations than the CD or ITC experiments and thus depending on the binding constants can be in a different binding regime (i.e., equilibrium vs. stoichiometric). The changes in the Trp fluorescence intensity upon titration of melittin with Ca^{2+} -S100P shown in Figure 6 indicate that the interactions are close to equilibrium. Thus it should be possible to analyze the titration profile using the binding formalism. The results of the CD and ITC experiments indicate that the stoichiometry of the melittin/ Ca^{2+} -S100P complex formation is one molecule of melittin per S100P monomer or because S100P exists as a homodimer (Gribenko and Makhataдзе 1998), two molecules of melittin per Ca^{2+} -S100P dimer. Figure 6 shows the results of the nonlinear regression analysis of the equilibrium binding of melittin to Ca^{2+} -S100P using equation 7. This equation describes the binding model that involves two identical and independent sites, assuming that each site contributes equally to the observed changes in fluorescence intensity. The fitted curve poorly (sum of square residuals 0.7) describes the shape of the titration profile indicating possible invalidity of this model. The use of the model that involves two different independent sites appears to be unjustified because of the symmetry consideration in the structure of S100P homodimer. However, based on the same symmetry consideration it is conceivable that the binding at one site of the homodimer involves conformational changes in the tertiary structure that transduce through the dimer interface and affect binding at the other site (i.e., the system is cooperative). Indeed the fit of the titration curve to equation 6 is clearly better (sum of square residuals 0.1) than the two-independent-sites model. Based on these results it appears that the binding of melittin to the dimer of Ca^{2+} -S100P is accompanied by positive cooperativity: the macroscopic binding constants for the binding to the first and second sites, K_1 and K_2 are $5 \pm 2 \mu\text{M}$ and $6 \pm 2 \mu\text{M}$, respectively. This can be translated into the cooperative energy as $\Delta\Delta_{coop} = -RT\ln 4(K_2/K_1)$ and is equal to $-4 \pm 1 \text{ kJ/mole}$, which indicates moderate positive cooperativity (Di Cera 1995).

Model for Ca^{2+} -dependent target recognition by S100P

According to the results presented above, S100P interacts with the amphipathic molecule of melittin, and, therefore, has a potential to interact with other proteins. These interactions are affected by Ca^{2+} . We have shown that in the presence of Ca^{2+} , binding of melittin to S100P results in formation of a tightly bound complex (K_d is within micromolar range) with the stoichiometry of two melittin molecules bound per S100P dimer. Furthermore, our results indicate that melittin binding is characterized by a positive cooperativity. Upon binding to Ca^{2+} -S100P, the target peptide adopts a helical conformation. Negative changes in heat

capacity upon melittin binding to S100P in the presence of Ca^{2+} and changes in fluorescence are indicative of Trp 19 burial and support the hypothesis that Ca^{2+} -dependent binding of melittin to S100P involves hydrophobic interactions.

The experiments presented here reveal the complexity of the possible S100P (and probably some if not all of the other S100 proteins) mediated signaling. An increase in calcium concentration up to micromolar levels (as observed during Ca^{2+} signaling in vivo) results in saturation of the C-terminal Ca^{2+} -binding site and leads to conformational changes necessary for the specific interaction of S100P with the target (e.g., melittin). We have shown previously that the binding of Mg^{2+} at the N-terminal binding site increases the Ca^{2+} -binding affinity at the C-terminal site (Gribenko and Makhatadze 1998). This would make the interaction of S100P with the target (melittin) possible at lower Ca^{2+} concentrations. The homodimer of Ca^{2+} -S100P can bind two target molecules that are either already in the helical state or fold into the helical structure upon binding to S100P. The binding of the first target molecule induces conformational changes that produce moderate positive cooperativity for the binding at the second site. The cooperativity in target recognition by S100 proteins may have important physiological implications. One can imagine that ability of some S100 proteins to form heterodimers (e.g., S100A/S100B; Baudier and Gerard 1986), S100A8/S100A9 (Hunter and Chazin 1998; Yang et al. 1999), S100P/S100Z (Gribenko et al. 2001) can further broaden the impact of cooperativity on recognizing two different targets. Furthermore, the receptor for one of the S100 proteins, S100A12, has been recently identified (Hofmann et al. 1999). Because the central event in receptor signaling often involves dimerization of the receptor, cooperative binding to two subunits will be an important factor.

Materials and methods

Purification of S100P and melittin

Recombinant S100P protein was overexpressed in *Escherichia coli* and purified as previously described (Gribenko et al. 1998). Melittin was purchased from Sigma and purified using Sephasil Peptide C18 reverse-phase column connected to an AKTA (Pharmacia) chromatography system as described by Brokx et al. (2001).

Isothermal titration calorimetry

The ITC experiments were performed in duplicate using a VP-ITC titration microcalorimeter (MicroCal, Inc.). The procedure for these experiments has been described previously (Lopez et al. 1999, 2001; Brokx et al. 2001). In brief, most of the experiments were performed by injecting 7–10 μL of S100P protein with concentrations ranging between 0.57 and 1.36 mM into the sample cell containing the melittin solution. The melittin concentration in the cell varied between 0.017 and 0.075 mM, depending on the magnitude of the heat effects observed. The concentrations used for the

reversed titration (melittin injected and S100P protein in the cell) were 1.9–3.0 mM and 0.022–0.08 mM, respectively. Dilution effects were taken into account by injecting titrant into the buffer. The heat of the reaction after each injection, Q_i , was obtained by integrating the peaks after each injection according to the ORIGIN software provided by the manufacturer. The binding isotherm was calculated by summing the individual heat effects and dividing it by the total number of moles of the specie in the ITC cell. When the stoichiometry of the binding reaction is one molecule of melittin bound to one molecule of S100P monomer, the enthalpy of binding, ΔH_o , was independent of whether the enthalpy is expressed per mole of specie in the cell or per mole of titrant, as was expected. The binding isotherms were fitted simultaneously to a binding equation (Brokx et al. 2001):

$$Q = \Delta H_o \frac{A - \sqrt{A^2 - 4 \cdot N_b \cdot [\text{cell}]_{\text{Total}} \cdot [\text{titrant}]_{\text{Total}}}}{2 \cdot [\text{cell}]_{\text{Total}}} \quad (1)$$

where $A = [\text{cell}]_{\text{Total}} + N_b [\text{titrant}]_{\text{Total}} + K_d$, $[\text{cell}]_{\text{Total}}$ and $[\text{titrant}]_{\text{Total}}$ are the total concentrations of species in the cell and in the syringe, respectively, Q is cumulative heat per mole of the cell species, N_b is the stoichiometry of melittin S100P complex, and K_d is the dissociation constant for the interaction.

CD spectroscopy

CD experiments were performed on a Jasco J-715 automatic recording spectropolarimeter as described (Gribenko and Makhatadze 1998). Far-UV CD spectra were measured in a 1-mm rectangular quartz cell. The buffer used was 5 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 3 mM DTT, with varying concentrations of calcium ions. The protein concentration in all samples was 30 μM , the melittin concentration ranged from 0 to 90 μM . To determine the changes in melittin ellipticity in complex with S100P with respect to free melittin ($\Delta\Theta$), the sum of the CD signal of protein alone and CD signal of free melittin at a given concentration was subtracted from the CD signal of the S100P-melittin complex. The molar ellipticity, $[\Theta]$, was calculated as

$$[\Theta] = \frac{\Delta\Theta \cdot M_w}{10 \cdot l \cdot C} \quad (2)$$

where M_w is the mean molecular mass of amino acid residues in melittin, C is the concentration of melittin in the solution in mg/mL, and l is the optical path length in centimeters. All experiments were performed in triplicate and the average values are reported.

Fluorescence spectroscopy

Steady-state fluorescence experiments were performed on a FluoroMax Spectrofluorimeter with DM3000F software (SPEx Industries, Inc.) as described previously (Gribenko et al. 1998; Lopez et al. 1999). A constant temperature in the thermostated cell holder (25°C) was maintained using circulating water bath. A quartz cell with a 1-cm path length was used. The buffer used in all titration experiments and in measurements of fluorescence emission spectra

was 25 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 3 mM DTT. Fluorescence emission spectra were recorded using an excitation wavelength of 295 nm, step resolution of 0.5 nm, and an integration time of 3 sec. For the titration experiments, the excitation and emission wavelengths were 295 and 353 nm, respectively. Titration of melittin solution (5 μ M) with S100P (0–25 μ M) was performed at 25°C, and the initial volume of melittin in a sealed fluorescence cell was 1.25 mL. Small aliquots of concentrated solution of S100P were added into the cell containing melittin solution. The solution in the cell was gently stirred during the titration and the intensity values were corrected for dilution; inner filter effect and blank backgrounds were subtracted. During the titrations the highest reliable ratio of S100P/melittin of 5:1 was possible to obtain. Higher S100P/melittin ratio was producing a large increase in the fluorescence of the background (buffer plus S100P). Backgrounds with high fluorescence intensity as compared to the intensity of the sample will affect the shape of the titration profile. In our experiments we have used only the data points for which the fluorescence intensity of the background is <5% of the intensity of the sample.

Acrylamide titrations were performed using 5 μ M solutions of melittin in the presence or absence of 25 μ M of S100P and/or 5 mM Ca^{2+} . The final acrylamide concentration was 0.5 M. Data were fitted to the modified Stern-Volmer equation (Eftink and Ghiron 1976):

$$\frac{I}{I_0} = (1 + K_{sv} \cdot [Q]) \cdot e^{V[Q]} \quad (3)$$

where I_0 is initial intensity of the fluorescence signal, I is fluorescence intensity at any given titration point, $[Q]$ is acrylamide concentration at any given titration point, V is the constant describing the static component of the quenching reaction, and K_{sv} is the Stern-Volmer constant and is a product of rate constant for quenching, k_q , and lifetime of the fluorophore τ_0 (Eftink and Ghiron 1976).

All fluorescence experiments were run in triplicate, intensity was corrected for dilution, and average values are reported.

Data analysis

Experimental observable changes in fluorescence intensity, $I/I_{\max}$, as a function of total ligand concentration, L_T , in the presence of macromolecule at concentration, M_T , are related to the concentration of the bound ligand, L_B , as

$$\frac{I}{I_{\max}} = \frac{L_B}{L_T} \quad (4)$$

The concentration of bound ligand can be related to the concentration of free ligand, $L_F = L_T - L_B$, the exact relation depends on the mode of interactions. We considered two different models. Due to the symmetry of the S100P homodimers, a model with two different independent sites was not considered (Wyman and Gill 1990; Di Cera 1995).

Model 1: There are two identical and independent binding sites.

$$\frac{L_B}{M_T} = 2 \cdot \frac{K_1 L_F}{1 + K_1 L_F} \quad (5)$$

Model 2: The general situation when there are two nonidentical and independent binding sites.

$$\frac{L_B}{M_T} = \frac{K_1 L_F + 2K_1 K_2 L_F^2}{1 + K_1 L_F + 2K_1 K_2 L_F^2} \quad (6)$$

Model 1 has an analytical solution that allows direct nonlinear regression analysis of the experimental observable using equation

$$\frac{I_i}{I_{\max}} = \frac{(2L_T + M_T + K_1) - \sqrt{(2L_T + M_T + K_1)^2 - 8L_T M_T}}{4 \cdot M_T} \quad (7)$$

Model 2 does not have analytical solution and was solved numerically. We used the Newton-Raphson method as implemented by Ababou and Desjarlais (2001). The quality of the fit to models 1 and 2 was assessed by the sum of squared residuals.

Δ ASA calculations

Δ ASA values were computed using the predicted three-dimensional structure of S100P in complex with melittin described in this study. The calculations were done according to Makhatadze and Privalov (1995) as described recently (Brokx et al. 2001). Briefly, the changes in surface areas are divided into four types: ASA_{bb} , the surface area of the backbone atoms; ASA_{arm} , the aromatic surface area; ASA_{alp} , the surface area for aliphatic groups; and ASA_{pol} , the surface area for polar groups. The changes in each type of surface area upon formation of the S100P-melittin complex, Δ ASA, were calculated as:

$$\Delta ASA = ASA_{S100P-Melittin} - ASA_{S100P} - 2ASA_{Melittin} \quad (8)$$

where $ASA_{S100P-Melittin}$, ASA_{S100P} , and $ASA_{Melittin}$ are the water-accessible surface area of the S100P-melittin complex, the free S100P, and the unstructured melittin, respectively. Structural modeling of the S100P dimer complexed with melittin was done using PDB entries 1BT6 (Rety et al. 1999) and 1QLS (Rety et al. 2000) as described previously (Gribenko and Makhatadze 1998; Brokx et al. 2001). The changes in the accessible surface area upon complex formation were used to calculate the expected heat-capacity change (ΔC_p) for this interaction using the empirical relationship (Brokx et al. 2001)

$$\Delta C_p = 2.14 \cdot \Delta ASA_{alp} + 1.55 \cdot \Delta ASA_{arm} - 1.81 \cdot \Delta ASA_{bb} - 0.88 \cdot \Delta ASA_{pol} \quad (9)$$

The numerical coefficients are expressed in J/(kmole $\text{\AA}^2$).

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