Two Conserved Residues Govern the Salt and pH Dependencies of the Binding Reaction of a PDZ Domain*

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PDZ domains are protein-protein interaction modules found in hundreds of human proteins. Their binding reactions are sensitive to variations in salt and pH but the basis of the respective dependence has not been clear. We investigated the binding reaction between PSD-95 PDZ3 and a peptide corresponding to a native ligand with protein engineering in conjunction with stopped-flow and equilibrium fluorimetry and found that the two conserved residues Arg-318 and His-372 were responsible for the salt and pH dependencies, respectively. The basis of the salt-dependent variation of the affinity was explored by mutating all charged residues in and around the peptide-binding pocket. Arg-318 was found to be crucial, as mutation to alanine obliterated the effect of chloride on the binding constants. The direct interaction of chloride with Arg-318 was demonstrated by time-resolved urea denaturation experiments, where the Arg-318 → Ala mutant was less stabilized by addition of chloride as compared with wild-type PDZ3. We also demonstrated that protonation of His-372 was responsible for the increase of the equilibrium dissociation constant at low pH. Both chloride concentration and pH (during ischemia) vary in the postsynaptic density, where PSD-95 is present, and the physiological buffer conditions may thus modulate the interaction between PSD-95 and its ligands through binding of chloride and protons to the “molecular switches” Arg-318 and His-372, respectively.

In the post-genomic era there will be ever-increasing focus on protein-protein interactions (1–3) because of their critical role in cellular function and the growing sequence and structure databases. Indeed, most processes in the cell are governed by more or less specific protein-protein interactions. Often the binding is tuned by cellular or extracellular conditions such as pH, ionic strength, and molecular crowding, and also by specific molecules acting allosterically on the protein or competing with the “natural” ligand. Many proteins or protein domains, have evolved to interact with other proteins and form complexes, and one of the most common of these protein-protein interaction modules is the PDZ domain, which is present in several hundred human proteins (4, 5). PDZ domains are often found within multi-domain scaffolding proteins, and they bind mainly to the C termini of their target proteins (6–8) but also internally (9, 10). PDZ domains have been shown to be rather promiscuous with regard to their ligand, and they have overlapping ligand specificities (6, 7).

One of the most well studied PDZ domains is PDZ3 from PSD-95.3 The three-dimensional structure of this protein with and without its target ligand has been solved (11). The canonical PDZ domain consists of about 90–100 amino acids forming six β strands (βA to βF) and two α helices; αA and αB neatly arranged in a spherical structure (Fig. 1a). Peptide binding takes place between the βB strand and αB helix in an antiparallel manner as shown in Fig. 1a. As judged by the crystal structure, PDZ3 binds its target peptide, which ends Lys-Gln-Thr-Ser/Val-COO− (numbered −4 to 0 from left to right) via a number of interactive forces. A series of hydrogen bonds from the carboxylate-binding loop stabilize the C-terminal carboxylate in the binding pocket. In addition, the carboxylate of the C-terminal valine interacts with Arg-318 of PDZ3 via a water molecule (11). In other PDZ domains, the residue corresponding to the Arg-318 is either lysine or arginine (9, 12–14). The threonine at position 2 of the peptide points into a pocket containing the His-372 of αB. The crystal structure suggests that a hydrogen bond is formed between the N-3 nitrogen of His-372 and the hydroxyl group of the threonine (11) (Fig. 1b). This histidine residue is conserved among PDZ domains that recognize type I peptide ligands (i.e. with the three final residues being (Ser/Thr)-X-(Val/Leu), where X is any residue) (15).

The pH and ionic strength of the solution affect the affinity between PDZ domains and their ligands. The variation of the rate constants of PSD-95 PDZ3 binding to a peptide (dansyl-KQTCSV-COO−) with pH (mainly koff) and ionic strength (mainly koff) has previously been observed (16) but not explained. Another study addressed the variation of the equilibrium free energy on NaCl concentration for mouse α1-syn- trophin PDZ domain binding to target peptides (VKESLV-COO− and SIESDV-COO−) but did not find what caused the observed effect (14).

In this work we have explored the basis for the pH and salt dependence of the rate and equilibrium binding constants of PSD-95 PDZ3. Our results show that the observed variation in

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*This work was supported in part by grants from the Swedish Research Council, Magnus Bergvall’s Foundation, the Medical Faculty, Uppsala University, and the Department of Medical Biochemistry and Microbiology, Uppsala University. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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3 The abbreviations used are: PSD-95, postsynaptic density protein-95; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
on-rate constant seen in the PDZ3-peptide interaction with varying ionic strength (16) is not because of screening of long-range forces according to the Debye-Hückel theory (17). Instead we found that chloride ion exerts an inhibitory effect on the PDZ3-peptide interaction through direct interaction with Arg-318. In addition we show that His-372 is responsible for the pH dependence of the binding reaction.

EXPERIMENTAL PROCEDURES

Numbering of the residues in PDZ3 wild-type and mutants is as in the crystal structure (11). The cDNA for wild-type PDZ3 (16), coding for residues 308–399 of PSD-95, was subcloned into the BamHI and EcoRI restriction sites of a modified pRSET vector (Invitrogen) resulting in expression of a His-tagged PDZ3. This “wild-type” was either with or without a tryptophan in position 337 (previously referred to as position 31 (16)). The plasmids encoding the mutants R318A, R318A/F337W, E331A, H372A, E373A, E373A/F337W, and K380A were made by inverted PCR using Pfu Turbo polymerase (Stratagene) and the wild-type PDZ3 cDNA as a template.

Expression and Purification of PSD-95 PDZ3 Wild-type and Mutants—BL21(DE3)pLysS competent cells (Invitrogen) were transformed with plasmid encoding wild-type PDZ3, the pseudowild-type F337W, and the mutants H372A, R318A, R318A/F337W, E331A, E373A, E373A/F337W, and K380A. The resultant transformants were grown on ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml) plates overnight at 37 °C. Colonies on the plate were used to inoculate 10 ml of pre-cultures, which in turn were used to inoculate 850 ml of 2× TY growth medium (16 g tryptone, 10 g yeast extract, 5 g NaCl/liter). The cultures were grown in a rotary shaker at 37 °C to an A600 of 0.45. Protein expression was then induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. Induced cultures were grown at 37 °C for three hours. Cells were harvested by centrifugation at 7500 × g for 5 min and resuspended in 50 mM Tris-HCl, pH 7.5. The resuspended pellets were ultrasonicated, centrifuged at 38,500 × g for 1 h and filtered through 0.22-μm filters. The filtered sample was loaded onto a nickel(II)-charged chelating Sepharose fast flow column (Amersham Biosciences), equilibrated with 50 mM Tris-HCl, pH 7.5, and washed with 300 ml of the same buffer. The bound protein was eluted with 250 mM imidazole, pH 7.9, in aliquots of 9 ml. Fractions containing PDZ were then filtered, diluted three times, and loaded onto a Q-Sepharose ion-exchange column (Amersham Biosciences). Elution was done with a gradient of 0–500 mM NaCl in 50 mM Tris-HCl, pH 7.5. Fractions contain-
ing PDZ domain were pooled together, and their purity was checked by SDS-PAGE. The identity of the pooled sample was acknowledged by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. The concentration of the PDZ domain was determined by absorbance measurements using extinction coefficients determined by amino acid analysis. Far UV circular dichroism studies on a Jasco J-810 spectropolarimeter and urea denaturation (for R318A/F337W and E373A/F337W) were performed to verify that the purified PDZ domains were folded.

**Pre-steady State and Equilibrium Measurements**—The peptide KQTSVcoo- that corresponds to the C terminus of the protein CRIP2 was previously synthesized with a dansyl group at the N terminus as described (16). The following buffers were used in the binding experiments: potassium acetate, pH 5.7 and Tris-HCl, at pH 8.5 (25 °C) or pH 9.0 (10 °C).

The salt dependence of the binding rate and equilibrium constants was determined in presence of different potassium salts: chloride, fluoride, potassium phosphate, and nitrate in separate experiments. Equilibrium measurements were performed by measuring the increase in dansyl fluorescence upon binding at 25 °C, pH 8.5, in an SLM 4800 spectrofluorimeter (SLM Instruments, IL). Excitation was at 345 nm and emission at 551 nm. The salt dependence of the binding rate and equilibrium constants was determined by measuring the increase in dansyl fluorescence upon binding at 25 °C, pH 8.5, and Tris-HCl, at pH 8.5 (25 °C) or pH 9.0 (10 °C).

The fluorescence of the dansyl group was not affected by the addition of salt. Experimental procedures for pre-equilibrium binding kinetics were as described previously (16). In brief, all measurements were done at 10 °C at pH 9.0 using Tris-HCl buffer on an SX-18MV stopped-flow spectrometer (Applied Photophysics, Leatherhead, UK). Fluorescence was monitored using either the increase in dansyl emission (excitation at 345 nm, emission > 475 nm) or the decrease in tryptophan emission (excitation at 280 nm, 465 nm > emission > 320 nm), when using both the tryptophan in the protein and the dansyl on the peptide as probes in the case of the pseudo-wild-type PDZ3/F337W (16), R318A/F337W, and E373A/F337W. Kinetic unfolding and refolding experiments were performed in the stopped-flow by mixing protein with buffer-urea solutions and monitoring the change in emission around 330 nm (bandpass filter) upon excitation at 280 nm. Experimental unfolding and refolding traces followed single exponential kinetics.

**Data Analysis**—Chloride was analyzed as being a competitive inhibitor of the peptide according to Scheme 1. P is the PDZ3, L1 is the peptide, and L2 is the chloride. KCl was added to PDZ3-peptide complex (5 and 10 μM, respectively), and the resulting fluorescence was recorded at equilibrium. Data from this assay were fitted to Equation 1.

**RESULTS**

The PDZ used in this study was similar to that in Gianni et al. (16), except that the present construct contained eight extra residues (MHHHHHPPR) at the N terminus. The stability of these His-tagged variants was similar to that of the previously used PDZ3 domain, which lacked the His-tag, as judged from urea denaturation curves and circular dichroism studies (not shown). Moreover, two variants were used as wild type, one with an engineered tryptophan (PDZ3/F337W) and the other without (PDZ3). The Trp in position 337 acts both as a fluorescent probe in the case of the pseudo-wild-type PDZ3/L1/HL1022.

### Scheme 1

\[
P + L_1 \overset{\text{PL}}{\rightarrow} P \cdot L_1 + L_2
\]

**Salt and pH Dependence of PDZ-Ligand Interactions**

\[
F = \left[ (K_D + K_{D2}[L_2]/K_{D2} + [P]_n) + [L_2]_0/2 - (K_D + K_D[L_2]/K_{D2} + [P]_n) + [L_2]_0/2 - (K_D + K_D[L_2]/K_{D2}) \right] \times B + C \quad (\text{Eq. 1})
\]

\[
F \text{ is the observed fluorescence signal, } K_D, K_{D2}, \text{ and } K_{D2} \text{ are the dissociation constants for } L_1 \text{ and } L_2, \text{ respectively, } [P]_n \text{ is the total concentration of PDZ3 (held constant), } [L_1]_0 \text{ is the total concentration of dansylated peptide (held constant), and } [L_2]_0 \text{ is the concentration of chloride (which was varied). } B \text{ and } C \text{ are the constants that take into account the total fluorescence change and endpoint, respectively (16). } K_D \text{ for the PDZ3-peptide interaction was determined independently (according to Equation 2) and used in the curve fitting. Data from equilibrium binding experiments were fitted to Equation 2.}
\]

\[
F = \left[ ([A]_0 + K_D + n)/2 - ([A]_0 + K_D + n)^2/4 - [A]_0 n)^{0.5} \right] \times B + C \quad (\text{Eq. 2})
\]

\[
F \text{ is the observed fluorescence signal, } n \text{ and } [A]_0 \text{ are the total concentrations of the nonvaried and varied species, respectively, and } K_D \text{ is the equilibrium dissociation constant. } B \text{ and } C \text{ are as stated for Equation 1.}
\]

Time-resolved fluorimetric traces from PDZ-peptide binding kinetics were fitted to a simple exponential function (Equation 3), where A is the signal recorded with time t. A_{EQ} is the total product formed at equilibrium, and k_{obs} is the observed rate constant.

\[
A = \Delta A_{EQ}(1 - e^{-k_{off}t}) + C \quad (\text{Eq. 3})
\]

The k_{obs} values were plotted versus PDZ concentration and fitted to the general equation for reversible association of two molecules, Equation 4 (16, 18).

\[
k_{obs} = \left( k_{on}^2 (n - [A]_0)^2 + k_{off}^2 + 2 k_{on} k_{off} (n + [A]_0) \right)^{0.5} \quad (\text{Eq. 4})
\]

k_{on} is the association or on-rate constant, k_{off} is the dissociation or off-rate constant, and [A]_0 and n are the initial concentrations of the varied and constants species, respectively. The dependence of the on-rate constant k_{on} on salt concentration was analyzed according to Equation 5.

\[
k_{on}^{app} = k_{on} \times 1/(1 + [\text{chloride}]/K_{D2}^{\text{chloride}}) \quad (\text{Eq. 5})
\]

k_{on}^{app} is the apparent rate constant at a certain chloride concentration, and k_{on} is the rate constant in absence of chloride. Fitting of the data were performed using Kaleidagraph version 4.0 (Synergy software).
resonance energy transfer donor in binding experiments with dansylated peptide. We have previously demonstrated that the binding kinetics of the PDZ3/F337W variant is similar to those of wild-type PDZ3 (16). The binding constants of the His-tagged PDZ3 were also similar to those obtained using PDZ3 without His tag. The numbering of residues in this paper is in accordance with the crystal structure of PSD-95 PDZ3 (11) (Protein Data Bank code 1BE9).

Effects of Different Anions on the Interaction—We found previously that the association rate constant, $k_{on}$, for the PDZ3-peptide binding reaction decreases as the ionic strength of the buffer is increased (16). As a first step toward determining whether the effect of ionic strength on the on-rate constant of the wild-type PDZ3 is actually because of screening of long-range charge-charge interactions as stipulated by Debye-Hückel theory or whether binding is hindered by specific interaction of the anion with the protein, competition binding assays at equilibrium were performed, where the respective salts KNO₃, KCl, KF, and potassium phosphate (mainly K₂HPO₄) were added to dissociate the PDZ3-peptide complex (Fig. 2). Surprisingly, KNO₃ and KCl dissociated the complex but KF did not, at least not to the same degree, and K₂HPO₄ even increased the signal slightly. We then performed pre-equilibrium binding experiments with three different anions: chloride, fluoride, and phosphate. Wild-type PDZ3 was rapidly mixed with dansylated peptide in the stopped-flow spectrometer, and the resultant change in fluorescence was recorded with time. The concentration of PDZ3 was varied at a constant peptide concentration, and observed rate constants, $k_{obs}$, were obtained by fitting a single exponential function to the data (16). These experiments were repeated at different constant concentrations of the three anions tested (Fig. 3). No or little effect on the on-rate constant was seen with fluoride and phosphate even when the ionic strength of phosphate was increased up to 2 M (Fig. 3). The hydration energy of the chloride ion is lower than that of fluoride (19). Fluoride therefore is hydrated by water to a higher degree, and the resulting ion is larger than the solvated chloride. Thus, chloride will penetrate the surface of the protein more than fluoride. The observed effect of the three different anions on the on-rate and calculated equilibrium constants ($k_{off}/k_{on}$) was of the order Cl⁻/HPO₄²⁻/F⁻/H₂PO₄⁻, i.e. the largest ion had the smallest effect. These results indicated that the chloride ion might be competing with the ligand for binding to PDZ3 and that long-range electrostatic forces did not medi-

![FIGURE 2. The effect of different anions on the PDZ3-peptide complex. Experiments were done on a spectrofluorimeter starting with an equilibrium mixture containing 5 μM PDZ3 and 10 μM peptide (total volume and concentrations of PDZ3 and peptide were kept constant throughout the experiment). Aliquots were withdrawn from the cuvette, and solutions of KCl, KF, KNO₃, or potassium phosphate (containing PDZ3 and peptide) were added and the decrease in fluorescence because of PDZ3-peptide dissociation recorded. The apparent $K_D$ values of the chloride and nitrate ions by this method were 200 ± 40 mM and 120 ± 10 mM (fitting errors), respectively, by fitting data to a competition model (Scheme 1 and Equation 1) as described under “Experimental Procedures.”](image)

![FIGURE 3. Variation of binding rate constants of wild-type PDZ3 and peptide to different anions. a, varying amounts of PDZ3 were rapidly mixed with a constant amount of peptide, and the resultant change in fluorescence was recorded with time. Observed rate constants, $k_{obs}$, were obtained by fitting a single exponential function to the data (Equation 3). Equation 4 was fitted to $k_{obs}$ versus PDZ3 concentration to obtain $k_{on}$ and $k_{off}$ for the binding reaction. This experiment was then repeated at different salt concentrations. b, log $k_{on}$ versus the square root of the ionic strength. There is an apparent linear dependence of $k_{on}$ when chloride is used as an anion. The effect on $k_{on}$ is small when fluoride and phosphate are used as anions, even at 2 M ionic strength in phosphate. c, log $k_{off}$ versus square root of the ionic strength. There is no dependence of the off-rate constants on chloride concentration.](image)
ate the binding of the peptide to PDZ3. The effect of chloride on $k_{on}$ rather than $k_{off}$ is easily explained if chloride acts as a competitive inhibitor that is in a fast pre-equilibrium with the PDZ domain. The observed on-rate constant $k_{on}^{app}$ would then equal the true $k_{on}$ multiplied by the unbound fraction of PDZ3 according to Equation 5 (see “Experimental Procedures”). The apparent $K_D$ of chloride for PDZ3 was determined as $360 \pm 60 \text{nM}$ by plotting the on-rate constant versus chloride ion concentration and fitting to Equation 5 (Fig. 4 and Table 1) in fair agreement with the $K_D$ determined from the competition experiment. However, the weaker effect of fluoride and phosphate on the rate and equilibrium constants may not be because of their inability of specific interaction with the protein but their higher kosmotropic (“structure making”) ability to stabilize the protein-ligand complex, as given by the Hofmeister series (20–22) (phosphate $> \text{fluoride} >$ chloride $> \text{nitrate}$).

**Mutagenesis of Charged Residues**—The apparent specific action of chloride and nitrate on the binding reaction suggested by the experiments prompted us to seek for the residue(s) involved in the anion binding and also investigate other charged residues that might influence binding of the peptide with its negative C terminus. All charged amino acid residues close to the binding pocket, based on the crystal structure (11), were therefore mutated to alanine to obtain the following mutants: R318A, R318A/F337W, E331A, E373A, E373A/F337W, and K380A. Arg-318 is linked to the carboxylate of the peptide C-terminal valine via an ordered water molecule (11). Glu-373 of the α-helix corresponds to Asp-143 of the mouse α1-syntrophin PDZ that is known to form a salt bridge with a lysine residue at position −4 of its peptide (23). Kinetic experiments similar to those for the wild type were done on the mutants (Table 2). In addition, equilibrium-binding experiments were performed by varying the concentration of PDZ3 wild type and mutants at a constant peptide concentration. Equilibrium data were fitted to Equation 2 to obtain a $K_D$ value for the respective interaction. The $K_D$ values of the different mutants varied roughly 3-fold, whereas the $k_{on}$ remained fairly constant on mutation (except for R318A) when the experiments were performed in buffer without addition of extra salt, giving the changes in free energy on binding, relative to the wild type as shown in Table 2. We went ahead to perform kinetic and equilibrium binding experiments of the different mutants at various [Cl$^-$]. We expected that if chloride exerted a direct action on a certain residue and if that interaction were the basis for the salt effect, then there would be less or no variation in the binding constants on chloride concentration for an alanine mutant. Equilibrium binding experiments corroborated the hypothesis; $K_D$ values appeared to increase with chloride concentration, except for the R318A mutant (Fig. 4a). But both accuracy and precision in the equilibrium constant were lower than for the rate constants, and in particular the on-rate constant $k_{on}$ was accurate and precise. The

**FIGURE 4. Relationship between binding constants and anion concentration for wild-type PDZ3 and mutants.** a, equilibrium dissociation constant, $K_D$ versus KCl. b, association rate constant, $k_{on}$ versus KCl. c, dissociation rate constant, $k_{off}$ versus KCl. Analyses in b were performed according to Equation 5. See Table 1 for $K_D$ values. Each point for the R318A/F337W in a is a mean and standard error of three separate experiments. The on and off-rate constants of the R318A/F337W mutant are virtually independent of chloride concentration.

| **TABLE 1** | **The equilibrium dissociation constant $K_D$ for chloride of wild-type PDZ3 and the mutants** |
|-----------------|---------------------------------------------------------------|
| **PDZ**         | $K_D^{\text{a}}$ | $K_D^{\text{b}}$ | $\Delta G^{\text{mut}}^{\text{b,c}}$ |
| Wild-type PDZ3  | $360 \pm 60$    | $200 \pm 40$    | $-$                                     |
| R318A/F337W     | $-4$            | $-4$            | $-4$                                     |
| E331A           | $250 \pm 50$    | $-4$            | $0.08 \pm 0.19$                          |
| E373A/F337W     | $230 \pm 40$    | $-4$            | $0.15 \pm 0.13$                         |
| K380A           | $580 \pm 160$   | $-4$            | $0.21 \pm 0.13$                         |

$^{a}$ The $K_D$ values were obtained by fitting on-rate constants at different chloride concentration into Equation 5.

$^{b}$ Competition assay; chloride was added to an equilibrium mixture of PDZ3 and peptide. $K_D$ for chloride was obtained according to Equation 1.

$^{c} \Delta G^{\text{mut}}$ for the R318A/F337W mutant could not be determined.

$^{d}$ Not determined.

| **TABLE 2** | **Rate and equilibrium parameters for the PDZ3-peptide interaction** |
|-----------------|---------------------------------------------------------------|
| **PDZ**         | $k_{on} \text{ (s}^{-1})$ | $k_{off} \text{ (s}^{-1})$ | $K_D (\mu M)$ | $\Delta G^{\text{mut}}^{\text{b,c}}$ |
| Wild-type PDZ3  | $5.5 \pm 0.4$        | $7.2 \pm 1.5$       | $1.3 \pm 0.3$ | $-4$                                     |
| R318A$^{c}$     | $2.2 \pm 0.1$        | $7.9 \pm 0.5$       | $3.6 \pm 0.3$ | $0.57 \pm 0.14$                          |
| E331A           | $6.5 \pm 0.4$        | $9.7 \pm 2.4$       | $1.5 \pm 0.4$ | $0.08 \pm 0.19$                         |
| E373A$^{c}$     | $6.5 \pm 0.2$        | $10.8 \pm 0.5$      | $1.7 \pm 0.1$ | $0.15 \pm 0.13$                         |
| K380A           | $4.2 \pm 0.1$        | $7.5 \pm 0.5$       | $1.9 \pm 0.1$ | $0.21 \pm 0.13$                         |

$^{a}$ Experiments were done with the tryptophan variant, that is F337W, R318A/F337W, and E373F/E373W, respectively.

$^{b} \Delta G^{\text{mut}}$ was calculated from the kinetic constants ($K_D \sim k_{off}/k_{on}$).

$^{c}$ The change in free energy on mutation when experiments were performed in buffer alone (i.e., no additional salt).
Salt and pH Dependence of PDZ-Ligand Interactions

**FIGURE 5.** Dependence of folding rate constants of R318A and wild-type PDZ3 on chloride concentration. a, unfolding rate constants $k_u$ for the wild-type and the R318A mutant at three different urea concentrations. b, refolding rate constants $k_f$ for the wild-type and R318A mutant at three different urea concentrations. The observed rate constant is given by $k_{obs} = k_u + k_f$ (s$^{-1}$), where $k_u$ and $k_f$ are the unfolding and refolding rate constants, respectively. At high urea, $k_{obs}$ is dominated by $k_u$ and at low urea by $k_f$. For the wild-type and R318A mutant identical at low and high salt demonstrating that the stabilizing effect of chloride is reflected in $k_u$ only. The wild type is stabilized more than the R318A mutant by salt (0.6 M KCl in 50 mM potassium phosphate) resulting in a change in free energy relative to the wild-type $\Delta \Delta G_{D,N}^{WT}$ of $-0.25$ kcal mol$^{-1}$.

Kinetic rate constants of the different mutants at different [Cl$^-$] are shown in Fig. 4. The $k_{off}$ of the R318A (or R318A/F337W) mutant was indeed constant ($\sim 2.0 \mu M^{-1} s^{-1}$) with increasing [Cl$^-$], whereas there were no significant differences between the chloride concentration dependencies of $k_{on}$ for the E331A, E373A/F337W, and K380A mutants and the wild-type PDZ3. The $k_{off}$ values on the other hand were rather constant with chloride concentration. These data suggest that Arg-318 is the residue responsible for the observed action of chloride. To further test this hypothesis we subjected wild-type PDZ3/F337W and the R318A/F337W mutant to kinetic unfolding and refolding experiments. Given the high degree of accuracy and precision of observed unfolding rate constants, even a small change in folding or unfolding rate constant can be considered to be significant. If Arg-318 binds chloride better in the folded than the unfolded state (which is expected for a specific binding site), chloride should stabilize the wild-type PDZ3 relative to the R318A mutant on denaturation. Experiments were performed for the wild-type PDZ3/F337W and R318A/F337W mutant at low (refolding conditions) and high (unfolding conditions) urea concentrations, respectively. Unfolding and refolding experiments were done both at 0 and 0.6 M KCl in 50 mM potassium phosphate, pH 7.45, at 25 °C in the stopped-flow fluorimeter. Briefly, in an unfolding experiment, protein in buffer was mixed with urea buffer solutions with or without KCl to final urea concentrations of 7.1–7.7 M. In a refolding experiment, urea-denatured protein was mixed with a urea buffer solution, with or without KCl, to final urea concentrations of 0.5–1.6 M. The unfolding rate constant $k_u$ for the wild-type PDZ3/F337W decreased more than that for the R318A/F337W mutant upon addition of salt. The refolding rate constants $k_f$ on the other hand were virtually identical for PDZ3/F337W and the R318A/F337 unfolding experiments for nitrate, fluoride, and phosphate (not shown). The results for all ions were identical in terms of $\Delta \Delta G_{D,N}^{WT}$ for both wild-type and R318A/F337W mutant at high and low salt (Fig. 5). The equilibrium constant for the folding of a protein is the ratio of its folding rate constants, i.e., $k_u/k_f$. Because $k_u$ remained constant on mutation, the ratio of their respective unfolding rate constants, $k_{mut}$ for PDZ3/F337W and R318A/F337W can be used to calculate the $\Delta G_{D,N}^{WT}$ for the mutation. At 7.4 M urea and low salt, $\Delta G_{D,N}^{WT}$ was approximately $-0.25$ kcal mol$^{-1}$, whereas $\Delta G_{D,N}^{WT}$ at 7.4 M urea and high salt was close to zero. These results show that the wild type is stabilized more by the addition of chloride than is the R318A/F337W mutant (by $-0.25$ kcal mol$^{-1}$) and corroborate that Arg-318 interacts directly with the chloride ion. To assess the nature of the interaction for other ions we performed identical refolding and
raises the $K_D$ approximately 100-fold at pH 8.5 showing the high impact of His-372 for the recognition of the ligand.

DISCUSSION

The PDZ domain is one of the most common types of protein-protein interaction domain in mammals (15, 24, 25). We demonstrate here that two conserved residues in PDZ3 of PSD-95, Arg-318 and His-372, can act as molecular switches to allow chloride (or other anions) and pH, respectively, to modulate the affinity of PDZ3 toward its ligand.

The negatively charged C-terminal carboxylate is bound via hydrogen bonds to the protein backbone NH groups of the so-called carboxylate-binding loop (11, 12, 23, 26, 27) (Fig. 1b). A positively charged residue (Arg-318 in PDZ3 and Lys-86 in mouse $\beta$-H9251 syntrophin PDZ) is however conserved in most PDZ domains (10, 11), and this residue is important for the affinity between PDZ domains and their ligands, as shown here and in Refs. 14 and 28. In PSD-95 PDZ3, Arg-318 coordinates a water molecule that in turn stabilizes the negative charge on the bound carboxylate, together with the backbone amides (11). Harris et al. (14) concluded that Lys-86 in $\alpha$1-syntrophin PDZ is not involved in a direct ionic interaction with the peptide carboxylate, in agreement with the published structures (11, 12, 23, 26, 27) as well as our data. The equilibrium constant of $\alpha$1-syntrophin PDZ domain binding to a C-terminal peptide is shown to display a salt dependence, with lower affinity at higher salt, similarly as PDZ3 in the present study (14). The salt effect on $\alpha$1-syntrophin PDZ was also dependent on the anion used, and again in accordance with our data, chloride modulated the affinity toward the peptide more than fluoride. Furthermore, mutation of the conserved positively charged residue in $\alpha$1-syntrophin PDZ (Lys-86 $\rightarrow$ Met) resulted in loss of affinity, as did the Arg-318 $\rightarrow$ Ala mutation in PSD-95 PDZ3. In contrast to the PDZ3-peptide interaction, the Lys-86 $\rightarrow$ Met mutation in $\alpha$1-syntrophin PDZ did not obliterate the dependence of the equilibrium dissociation constant on chloride concentration. Instead, the effect of chloride was amplified by a factor $\sim$2 by the mutation. Therefore, the basis of the salt dependence for $\alpha$1-syntrophin PDZ and PSD-95 PDZ3 is distinct. Whereas chloride interacts directly with Arg-318 in PSD-95 PDZ3 with a $K_D$ value of around 300 mM, there is no evidence for this interaction in $\alpha$1-syntrophin PDZ. Could the binding of, for example, chloride to Arg-318 in PSD-95 PDZ3 be of physiological importance? The intracellular concentration of chloride is close to 32 mM (29), whereas the extracellular chloride concentration is around 150 mM. In the postsynaptic density, where PSD-95 is present, the concentration of chloride could vary between 32 and 150 mM. A resting neuron is characterized by a large excess of chloride ions on the outside of the cell as compared with the inside. GABAergic inputs from horizontal cells, which act on sodium potassium chloride co-transporter, are present in the dendritic spines. These are capable of causing hyperpolarization of the neurons that results in an influx of chloride inside the cells (30). Depending on the effective concentration of ligand for PSD-95 (for example, CRIPT (13), Citron (31), Neuriligin (32)), the concentration of chloride may modulate the binding. If, for example, the total concentration of all different ligands for PSD-95 PDZ3 is 10-fold below their average $K_D$ value (weighted mean), a rise in chloride concentra-
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tion from 32 to 150 mM would indeed compete out a large fraction of bound ligands. If, on the other hand, PSD-95 PDZ3 is saturated with ligands; 150 mM chloride would not affect the binding because we estimate $K_D^c$ for chloride to be slightly higher than this.

Another conserved residue among PDZ domains is a His at the beginning of αB (His-372 in PDZ3). The His-372 → Ala mutant shows that this residue is responsible for the pH dependence of the binding of PDZ3 to its ligands. It is likely that all PDZ domains having Class I specificity (4, 15), with a Thr or Ser residue at -2 position of the peptide ligand, display a similar pH dependence because of titration of the conserved His residue. Changing the pH is thus another way to modulate PDZ-ligand interactions. We showed previously that the affinity between PDZ3 and the peptide ligand starts to decrease below pH 7.0 (16). The normal intracellular pH of brain cells is around 7.0, but following brain ischemia the extracellular pH can plummet down to 6 (ischemic acidosis) and subsequently cause a decrease of the intracellular pH, and this drop in pH is considered a major cause of the resulting injury (33). Even a drop of the pH to 6.5 would influence the affinity between PSD-95 PDZ3 and its ligands because of protonation of His-372. Such a dramatic decrease of pH would undoubtedly affect the function of PSD-95, whereas it is difficult to speculate about a physiological role for the pH dependence of $K_D$ under normal conditions.

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