PDZ domains are protein adapter modules present in a few hundred human proteins. They play important roles in scaffolding and signal transduction. PDZ domains usually bind to the C termini of their target proteins. To assess the binding mechanism of this interaction we have performed the first in-solution kinetic study for PDZ domains and peptides corresponding to target ligands. Both PDZ3 from postsynaptic density protein 95 and PDZ2 from protein tyrosine phosphatase-L1 bind their respective target peptides through an apparent A + B → A-B mechanism without rate-limiting conformational changes. But a mutant with a fluorescent probe (Trp) outside of the binding pocket suggests that slight changes in the structure take place upon binding in protein tyrosine phosphatase-L1 PDZ2. For PDZ3 from postsynaptic density protein 95 the pH dependence of the binding reaction is consistent with a one-step mechanism with one titratable group. The salt dependence of the association reaction but not for dissociation of the complex.

PDZ4 domains are found in a few hundred human proteins, either as a single domain or in arrays. These domains mediate binding to other proteins and in this way play important roles in scaffolding and signal transduction (1, 2). Structural studies have shown that the PDZ domains usually bind to the C terminus of their target proteins. A number of crystal and NMR structures of PDZ domains have been solved both with and without bound peptide (for example, Refs 3–6) (Fig. 1). A wealth of data on different peptides binding to different PDZ domains has been obtained by screening (for example, Refs. 7 and 8) and selection (for example, Refs. 9 and 10). Such studies and those using the yeast two-hybrid technique (for example, Ref. 11) provide important information on possible cellular targets for distinct PDZ domains as well as the specificity of the interaction. Moreover, theory and NMR experiments have suggested that the dynamics of PDZ domains and the residues outside of the binding pocket influence their interaction with ligands (12, 13). Despite considerable effort to clarify the structural basis for the PDZ-ligand interaction, only a handful of studies have assessed the binding energetics and specificity of PDZ-peptide interactions using proper equilibrium assays in solution (3, 11, 14–24). Kinetics of chemical reactions not only provide “end point data” such as equilibrium constants but also yield microscopic rate constants and, more importantly, the possibility of elucidating the mechanisms of binding and probing the binding dynamics as well as the properties of the transition state of the reaction. To assess the binding mechanism, we have performed the first kinetic study of PDZ domains in solution using stopped-flow fluorimetry. The PDZ domains chosen were PDZ3 from human PSD-95, one of the most well studied PDZ domains, and the second PDZ domain from mouse protein tyrosine phosphatase-L1 (PTP-BL; also known as PTPL1, PTP-BAS, PTP1E, FAP-1, or PTPN13).

MATERIALS AND METHODS

Expression and Purification of PSD-95 PDZ3—The cDNA encoding PDZ3 of human PSD-95 (residues 352–444) was cloned into the BamHI and EcoRI restriction sites of a modified pRSET vector (Invitrogen) (resulting in a clone expressing His-tagged PDZ3). The plasmid was used to transform Escherichia coli BL-21 (DE3) pLys (Invitrogen), and protein expression was initiated by inducing 800-ml bacterial cultures with 1 mM isopropyl-β-D-thiogalactopyranoside at an A600 of ~0.5. The cultures were grown overnight at 30 °C, and the bacteria were harvested by centrifugation. The bacterial pellet was resuspended in 50 mM Tris-HCl and 400 mM NaCl, pH 7.5, and the bacteria were lysed by ultrasonication. After centrifugation, the soluble fraction was passed through a 0.22-μm filter and loaded onto a nickel(II)-charged chelating Sepharose FF (Amersham Biosciences) column equilibrated with 50 mM Tris-HCl and 400 mM NaCl, pH 7.5. After washing with the same buffer, the His-tagged PSD-95 PDZ3 was eluted with 250 mM imidazole, pH 7.9. 20 units of thrombin (Amersham Biosciences) were added, and the sample was incubated at 37 °C overnight. The sample was then diluted 3-fold in water and loaded onto a Q-Sepharose column equilibrated with 50 mM Tris, pH 7.5. Bound protein was eluted with a linear 0–0.5 M NaCl gradient in 50 mM Tris, pH 7.5. Fractions containing pure PSD-95 PDZ3, as judged by SDS-PAGE, were pooled, and identity was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The PSD-95 PDZ3 used in the present study contained 95 residues, two non-native residues in the N terminus (Gly and Ser, resulting from the thrombin recognition and BamHI sites) and residues 352–444 from human PSD-95 (numbered 1–95). Expression, purification, and numbering of residues of mouse PTP-BL PDZ2 were as described previously (25).
Kinetics of PDZ-Ligand Interactions

Peptide Synthesis—The peptides EQSVEVQCOO– and EQSVEVQCOO– were synthesized in an ABI 431 peptide synthesizer (Applied Biosystems, Foster City, CA) using standard FastMoc chemistry and Val-Wang resin to obtain a free carbonate in the C terminus. A dansyl group was attached to the N termini of the peptides by incubating the peptide-containing resin with a ~4-fold excess of dansyl chloride (Sigma) and an 8-fold excess of N-ethylidissopropylamine over the peptide N-амино group in N,N-dimethylformamide. The peptides were deprotected, purified by reverse phase chromatography, and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Concentrations of peptide and protein solutions (and thus extinction coefficients) were determined by amino acid analyses.

Spectroscopic Experiments—Buffers for experiments were made by dissolving the appropriate amount of acid and base (potassium salt where applicable) in H2O (18 megohms resistance) and adjusting the ionic strength with potassium chloride. The following buffers were used at different pH values: acetate, 5.9–6.1; phosphate, 6.4–8.0; and Tris, 8.4–9. Urea-induced denaturation experiments for PSD-95 PDZ3 were performed in 50 mM potassium phosphate and 21 mM potassium chloride at 25 °C (I = 150 mM). The pH and urea denaturation of wild-type PSD-95 PDZ3 was monitored by far-UV circular dichroism in a Jasco J-810 spectropolarimeter. Urea denaturation of the F31W mutant of PSD-95 PDZ3 was monitored by following Trp fluorescence using the APP software and the Kaleidagraph version 3.6 package (Synergy Software).

Data Analysis—the equations for analysis of bimolecular reactions under second order conditions have been derived recently (27). The general equation for reversible association of two molecules is shown in Equation 1,

\[ A = \Delta A_{EO}(1 - e^{-k_{obs}t})/(1 + \omega e^{-k_{obs}t}) \]  

(Eq. 1)

where \( A \) is the amount or concentration of product formed at time \( t \), \( \Delta A_{EO} \) is the total product formed at equilibrium, and \( \omega \) a parameter that varies between −1 and 0, depending on how well the experiment is obeying pseudo-first order kinetics. For PDZ-peptide interactions it was found that \( \omega \) is close to 0, and Equation 1 breaks down to a single exponential. The dependence of the observed rate constant \( k_{obs} \) on ligand concentration for a reversible association reaction follows Equation 2,

\[ k_{obs} = \sqrt{k_1(n - [A]_0)^2} + k_{-1} + 2k_1k_{-1}(n + [A]_0) \]  

(Eq. 2)

where \( k_1 \) is the association or on-rate constant, \( k_{-1} \) is the dissociation or off-rate constant, and \([A]_0\) and \( n \) are the respective initial concentrations of the two reacting molecules. In a typical experiment one species is varied ([A]0), whereas the other is held constant (n). The concentration of the constant species \( n \) can either be fitted together with \( k_1 \) and \( k_{-1} \) (if the quality of the data is good enough) or can be set to the independently determined value. Under pseudo-first order conditions where, for example, \([A]_0 \gg n\), Equation 2 breaks down to the commonly used Equation 3 (26) shown here.

\[ k_{obs} = k_1[A]_0 + k_{-1} \]  

(Eq. 3)

End points from time-resolved fluorometric measurements were fitted to the standard quadratic equation for equilibrium binding shown in Equation 4,

\[ F = \frac{([A]_0 + K_D + n)/2}{\sqrt{([A]_0 + K_D + n)^2 - [A]_0n} × B + C} \]  

(Eq. 4)

where \( F \) is the observed fluorescence signal, \( n \) and \([A]_0\) are the total concentrations of non-varied and varied species, respectively, and \( K_D \) is the equilibrium dissociation constant. \( B \) and \( C \) are constants that take into account the total fluorescence change and fluorescence at \([A]_0 = 0\), respectively. The amplitudes of the kinetic traces were fitted to a similar equation, but assuming \( F = 0 \) (or more correctly \( \Delta F = 0 \)) at \([A]_0 = 0\), i.e. \( C \) in Equation 4 = 0.

The pH dependence of rate and equilibrium constants are often complex but can sometimes be approximated by the standard equation for one titratable group, which was the case for the off-rate constant \( k_{-1} \) as seen here in Equation 5,

\[ k = A \times \frac{[10^{(pK_a - pH)}/(1 + 10^{-(pK_a - pH)})]}{1 + [1/(1 + 10^{-(pK_a - pH)})]} \]  

(Eq. 5)

where \( A \) is the value of the pH-dependent constant at low pH and \( B \) is the value of this constant at high pH.

The dependence of the rate constants \( k \) on ionic strength was analyzed according to the Debye–Hückel theory (28, 29) as shown in Equation 6,

\[ \log k = \log k_0 + 2Cz_iz_\theta \lambda 0.5 \]  

(Eq. 6)

where \( k_0 \) is the value of the rate constant in absence of salt, \( C \) is a constant close to 0.5 (liter/mol)0.5 in aqueous solution (28), \( z_i \) and \( z_\theta \) are the respective charges of the interacting molecules or moieties, and \( I \) is the ionic strength of the solution (m). Fitting of the data were performed using the APP software and the Kaleidagraph version 3.6 package (Synergy Software).

RESULTS

Structure and Stability of the PDZ Domains—PDZ domains typically consist of six β-strands, βA–βF, and two α-helices, αA and αB (see Fig. 1). The PSD-95 PDZ3 used contained five residues N-terminal to the first β-strand βA (GSREP) and eight residues after the last strand βF (PEEYSRFE). (The numbering of the residues in this paper starts with Gly-1 and ends with Glu-95.) The protein was thus slightly different from that crystallized by Doyle et al. (6) in that it did not contain an extra non-native C-terminal β-sheet and had fewer residues in the N terminus. By analyzing tryptic peptides with mass spectrometry we found that Asn-57 of PSD-95 PDZ3 and Asn-69 of mouse PTP-BL PDZ2 were deamidated, but it is difficult to determine whether the deamidation takes place before or after trypsin digestion. The Asn residues are followed by a Gly, which is a sequence that is prone to deamidation (30).
Because the Asn residues are solvent-exposed and situated in the turn following βD, far from the peptide-binding site, we find it unlikely that the possible deamidation affects ligand binding. Circular dichroism spectra at different pH values and urea denaturation (Fig. 2) showed that both the wild-type and F31W mutant of PSD-95 PDZ3 were folded under the conditions of the binding experiments. The extrapolated stability at pH 7.4 and 25 °C was 6–6.5 kcal mol⁻¹ in buffer without urea for both wild-type and F31W PSD-95 PDZ3. The stability of mouse PTP-BL PDZ2 was previously determined as 3 kcal mol⁻¹ under the experimental conditions (31). Folding rate constants should therefore not affect the observed rate constants for ligand binding.

**Ligand Binding** —The dansylated peptide D-KQTSV, corresponding to the C terminus of the protein CRIP (11), was used with PSD-95 PDZ3 in stopped-flow binding experiments. Upon binding of the peptide to PSD-95 PDZ3 the fluorescence of the dansyl group was increased (λ_em = 345 nm; λ_exc > 420 nm), and time-resolved binding traces could be recorded (Fig. 3). In binding experiments with PTP-BL PDZ2, the dansylated peptide used was D-EQVSAV, corresponding to the C terminus of the guanine nucleotide exchange factor RA-GEF-2 (32) and shown previously to interact with human PTP-BL PDZ2 (4). The length of the peptide used may influence the affinity. In this work we designed the peptides based on their interactions with the respective PDZ domain in the crystal (6) or NMR (4) structure. Also, to get a change in fluorescence upon binding, the dansyl group must be close to the protein in the bound state. However, the peptide D-EQVSAV did not display a change in dansyl fluorescence upon binding to PTP-BL PDZ2 (λ_em = 345 nm; λ_exc > 475 nm). To obtain a second probe for ligand binding, a mutation in βD was employed, namely Phe-31 (PSD-95 PDZ3) or Tyr-43 (PTP-BL PDZ2) → Trp (see Fig. 1). A Trp in this position was previously shown to function as a probe for protein folding studies (31, 33). In PSD-95 PDZ3 the side chain of the Phe-31 is stacked between βB, the C-terminal helix (mainly Glu-90, Tyr-91, and Phe-94), and βD (mainly Gln-52) (Fig. 1). No part of Phe-31 is in direct contact with the peptide, but its CB and backbone atoms make contacts with Ile-21 and Val-22 that, in turn, interact with the bound peptide (6). The NMR structure of human PTP-BL PDZ2 and bound peptide (4) shows that the side chain of the Tyr residue in the corresponding position is partially solvent-exposed. The main interactions of the Tyr side chain are with Val-44 (βC) and Val-37 (loop connecting βB and βC) and Gln-62 Arg-64 at the beginning of βD (numbering according to Walma et al.) (25). Again, Tyr-43

**Kinetics of PDZ-Ligand Interactions**

![Figure 1. Structure of PSD-95 PDZ3 with bound peptide (red stick, with C-terminal carboxylate) solved by Doyle et al. (6). The Phe-31 that was mutated to Trp is shown in green. (The residue in the corresponding position of wild-type PTP-BL PDZ2 is Tyr-43.) Arg-12, Ile-21, Gln-52, His-66, Tyr-91, and Phe-94 are shown in gray. (See “Results” for numbering.) The PSD-95 PDZ3 used in the present work ended at Glu-95 and did not contain the C-terminal two β-strands present in the crystal structure nor the most N-terminal residues in the picture. The picture was drawn in PyMOL (38).](http://www.jbc.org/)

![Figure 2. Stability of PSD-95 PDZ3. All experiments were performed at 25 °C with l = 150 mm and pH of 7.4 unless otherwise indicated. A, far-UV circular dichroism spectra of wild-type PSD-95 PDZ3 at different pH values. HT reflects the amplification of the measured signal and should be below ~700 V to give reliable data. B, circular dichroism-monitored urea denaturation of PSD-95 PDZ3 (left axis); m_DN = 1.09 ± 0.05 kcal mol⁻¹ M⁻¹, [urea]_em = 5.87 ± 0.03 μM, and ΔG_DN = 6.4 ± 0.2 kcal mol⁻¹ (fitting errors), assuming denaturant independence of the denatured state signal. Insert, circular dichroism spectra of wild-type PSD-95 PDZ3 at different [urea] values. Fluorescence-monitored urea denaturation of the F31W mutant of PSD-95 PDZ3 (right axis); m_DN = 1.06 ± 0.03 kcal mol⁻¹ M⁻¹, [urea]_em = 5.53 ± 0.02 μM, and ΔG_DN = 5.9 ± 0.08 kcal mol⁻¹ (fitting errors).](http://www.jbc.org/)
is not making direct interactions with the bound peptide, although its Cβ and backbone atoms make contacts with residues in βB (e.g. Val-29 and Thr-30) that, in turn, interact with the peptide.

The fluorescence of the introduced Trp was affected by ligand binding for PTP-BL PDZ2 Y43W (Fig. 4), but not for PSD-95 PDZ3 F31W, using an unlabeled ligand. Wild-type PSD-95 PDZ3 and the F31W mutant displayed similar rate constants for the interaction with D-KQTSV (Fig. 3). These experiments demonstrate that the F31W mutant displayed similar rate constants for the interaction with PTP-BL PDZ2 Y43W (Fig. 4), but not for PSD-95 PDZ3 F31W, Thr-30) that, in turn, interact with the peptide.

To Equation 2 where

\[
k_1 \text{mis}k_1 \text{mis}
\]

is the association rate constant.

The reported rate and equilibrium constants were fitted by constraining \( n \) in Equations 2 and Figs. 3–4. The on-rate constants \( k_1 \) for both PSD-95 PDZ3 and PTP-BL PDZ2 Y43W were in the range of \( 10^6 \)–\( 10^7 \) M\(^{-1}\) s\(^{-1}\) at 10°C, whereas the off-rate constants \( k_{-1} \) varied between 5 and 40 s\(^{-1}\) for PSD-95 PDZ3 and 40 and 70 s\(^{-1}\) for PTP-BL PDZ2 Y43W, depending on the pH value and salt concentration (Fig. 5 and TABLE ONE). Equilibrium dissociation constants (\( K_D \) values) calculated from the rate constants thus ranged from 0.6 to 7 μM, depending on the conditions. \( K_D \) values can be calculated from amplitudes and end points of the kinetic traces. The end points correspond to data obtained from an equilibrium binding experiment, given that there are no slow conformational changes occurring after collection of stopped-flow data. The \( K_D \) values for the PSD-95 PDZ3-peptide interaction obtained from kinetic amplitudes and rate constants were generally lower than the \( K_D \) values obtained from the end points of the kinetic traces (TABLE ONE and Fig. 6). The accuracy of the \( K_D \) values from rate constants are higher because the quadratic equation used for estimating \( K_D \) values for equilibrium titrations (Equation 4) is more dependent on a correct value of the non-varied species and is also less forgiving of scatter in the data.

The reported rate and equilibrium constants were fitted by constraining \( n \) in Equations 2 and Figs. 3–4. Including \( n \) as a variable, however, yielded

\[
\text{PDZ}+\text{pept} \xrightarrow{k_1} \text{PDZ}+\text{pept}
\]

values that were in good agreement with the concentration of the PDZ domain or the peptide calculated from absorption measurements (see legend to Fig. 3). This agreement serves as a good control of the data in general and the concentration determinations in particular (extinction coefficients were obtained by amino acid analyses). Note that an error in the concentration of the varied species gives a corresponding error in the association rate constant.
Kinetics of PDZ-Ligand Interactions

The pH and salt dependences of rate constants may yield useful information regarding the binding mechanism. The pH dependences for \( k_1 \) and \( k_{-1} \) were found to be qualitatively similar for PSD-95 PDZ3 and PTP-PL PDZ2 Y43W (Fig. 5). \( k_1 \) remained fairly constant for both PTP-PL PDZ2 Y43W and PSD-95 PDZ3. The \( k_{-1} \) for the PSD-95 PDZ3 binding reaction displayed a clear titration curve, and the \( k_{-1} \) for PTP-PL PDZ2 Y43W also increased as the pH was lowered.

Fig. 5B shows the ionic strength dependence of the interaction between wild-type PSD-95 PDZ3 and the dansylated peptide D-KQTSV at pH 8.45. The data were analyzed according to the Brønsted formalism (28, 29) as shown in Equation 6. Ionic strength affects primarily the on-rate constant \( k_1 \), which decreases from \( \sim 8 \times 10^6 \) to \( \sim 2 \times 10^6 \) M\(^{-1}\) s\(^{-1}\) as \( I \) is increased from 28 to 1000 mM, whereas the off-rate constant \( k_{-1} \) is almost constant in this range. Thus, the thermodynamic stability of the complex is controlled by the on-rate constant.

Amplitude Analysis—Analysis of the amplitudes of kinetic traces may reveal the presence of fast reactions that take place in the dead time of the stopped-flow instrument. For the reaction between PTP-PL PDZ2 Y43W and EQVSAV there was no evidence for fast formation of intermediate species before binding (Fig. 6A). In the majority of experiments with constant D-KQTSV and varied PSD-95 PDZ3, a small burst phase was detected (Fig. 6B).

DISCUSSION

This is the first study where rate constants for PDZ-peptide interactions were determined in solution. The observed kinetics suggest that PDZ domains bind their target proteins in a simple bimolecular reaction without rate-limiting conformational changes (Scheme 1). We cannot exclude a fast “docking” step subsequent to ligand encounter with a rate constant of 1000 s\(^{-1}\) or higher because of the limitations of the stopped-flow technique. In fact, such a step is likely to be present in most protein-ligand associations (34) and may be inferred from the present results for PTP-PL PDZ2 Y43W. The environment around the engineered Trp changes for PTP-PL PDZ2 Y43W upon binding of a ligand. As judged by the NMR structure (4) the ligand should not bind directly to the Trp, implying that some rearrangement of second sphere interactions does occur upon binding. This lends support to the notion that residues outside of the

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**TABLE ONE**

Rate and equilibrium constants of the binding reaction between PDZ domains and peptides

Parameters were obtained by fitting Equations 2 and 4 (\( I \) constrained) to experimental data collected at 10 °C and an ionic strength of 150 mM. A dash indicates no data.

| Constant species | Varied species | \( k_1 \) (M\(^{-1}\) s\(^{-1}\)) | \( k_{-1} \) (s\(^{-1}\)) | \( K_D \) (M) | \( K_D \) (amplitudes) | \( K_D \) (end points) | No. of independent binding curves |
|-----------------|----------------|----------------|----------------|-----------------|----------------|----------------|----------------|
| D-KQTSV\(^a\) | PSD-95PDZ3 (pH 7.45) | 7.9 ± 0.4 | 6.2 ± 0.5 | 0.8 ± 0.1 | 0.9 ± 0.3 | 2.4 ± 0.6 | 5 |
| D-KQTSV\(^a\) | PSD-95 PDZ3F31W (pH 7.45) | 8.1 ± 0.2 | 4.8 ± 0.4 | 0.6 ± 0.1 | 0.8 ± 0.1 | 2.8 ± 0.3 | 1 |
| D-KQTSV\(^a\) | PSD-95 PDZ3 (pH 8.45) | 5.3 ± 0.4 | 5.0 ± 0.2 | 0.9 ± 0.1 | 0.8 ± 0.1 | 1.5 ± 0.1 | 3 |
| PSD-95 PDZ3\(^b\) | D-KQTSV (pH 8.45) | 4.1 ± 0.2 | 4.6 ± 0.4 | 1.1 ± 0.1 | 1.4 ± 0.2 | —\(^d\) | 1 |
| PTP-PL PDZ2 Y43W\(^b\) | EQVSAV (pH 6.9) | 10 ± 2 | 72 ± 2 | 7.2 ± 0.3 | 5.0 ± 0.7 | 6 ± 2 | 1 |
| D-EQVSAV\(^b\) | PTP-PL PDZ2Y43W (pH 6.9) | 9.0 ± 1.4 | 54 ± 8 | 6.0 ± 1.3 | — | —\(^d\) | 1 |

\(^a\) ± S.E.
\(^b\) ± Fitting errors.
\(^c\) ± S.E.
\(^d\) Varying a fluorescent species complicates analysis of the end points.
binding pocket influence the interaction between PDZ domains and their ligands (12, 13). But direct evidence for a rate-limiting docking step can only be obtained by methods that can monitor faster reactions. Note that in the crystal structures of PSD-95 PDZ3 there are no significant differences between the peptide-bound and the unliganded protein (6), and no difference in fluorescence was detected in binding experiments with PSD-95 PDZ3 F31W and unlabeled peptide. In addition, the pH dependences of the rate constants for PSD-95 PDZ3 are consistent with a one-step mechanism (Figs. 5 and 7). It appears that a residue involved in binding of the peptide titrates with a $pK_a$ value of 5.8 in the PDZ-ligand complex (reflected in $k_{-1}$ and $K_a$). In free PSD-95 PDZ3 or peptide the residue titrates with a $pK_a$ of $\sim 6.9$ (reflected in $K_a$). The residue involved is possibly His-66 in helix $\alpha B$. In addition to van der Waals’ interactions, His-66 forms a hydrogen bond with the Thr hydroxyl group of the peptide (6).

Analysis of the kinetic amplitudes and end points for the Y43W mutant of PTP-BL PDZ2 and an unlabeled peptide (Fig. 6) showed that no spectroscopically visible events occurred in the “dead time” of the experiment, indirectly suggesting that no conformational changes took place in the protein prior to association of PDZ and peptide. Kinetic amplitudes and end points for the reaction between labeled peptide and “unlabeled” wild-type PSD-95 PDZ3, however, suggested the presence of an early event prior to the main association (Fig. 6B). Burst phase analyses are prone to errors and are critically dependent on a well determined time point for the actual mixing of the two solutions in the stopped flow. The question of whether the burst phase effect is due to nonspecific interactions of the protein with the dansyl group or is a real phenomenon, such as a conformational change of the peptide, has to await further studies. Direct detection of the apparent burst phase would help elucidate its true nature but would require faster techniques than stopped flow.

The on-rate constant $k_1$ decreased as the ionic strength was increased for the reaction between PSD-95 PDZ3 and $D$-KQTSV (Fig. 5B). The total charge of the peptide $D$-KQTSV is zero, and that of PSD-95 PDZ3 is negative. The random collision frequency should therefore not be affected by changes in ionic strength of the solution. The salt dependence indicates that the salt screens out ionic interactions between the PDZ domain and the ligand that are in the process of being formed in the transition state of the binding reaction. The slopes of these plots (i.e. $2C_{\text{z}_{\text{a}}^2}\varepsilon_{\text{a}} \approx 2C_{\text{z}_{\text{b}}^2}\varepsilon_{\text{b}}$, Equation 6) are non-zero and negative for $k_1$ ($-0.64$) and close to zero for $k_{-1}$ ($-0.05$), respectively. The negative slope suggests that the association reaction between PSD-95 PDZ3 and the pep-
tide is electrostatic and that long-range coulombic forces (varying with $1/r^2$) dominate the diffusional encounter. From the $z_6z_8$ term (approximately $-0.64$) of the on-rate constant we can estimate an average of $0.8$ charges of opposite sign on each binding partner. There are no obvious candidate residues to explain this result. Crystallographic and mutagenesis studies (e.g. replacing the C-terminal carboxylate with an alcohol (14)) have shown that the carboxylate of the C-terminal peptide residue (Val in this case) is crucial for the affinity of C-terminal peptides for PDZ domains and their ligands (14). In PDZ-95 PDZ3 there are two Glu residues, Glu-25 and Glu-67, that may form surface ionic interactions with the Lys residue of the peptide.

The $K_r$ values determined in this study ($\sim 1 \mu M$ for PSD-95 PDZ3 and $\sim 7 \mu M$ for PTP-BL PDZ2 Y43W at neutral pH; TABLE ONE) agree well with those obtained in PDZ-peptide equilibrium studies performed in solution using fluorescence (3, 14, 15) or fluorescence anisotropy (11, 17–19, 24). Because of discrepancies between in-solution and interaction studies on PDZ domains where one binding partner is immobilized, rate constants obtained using surface plasmon resonance must be used with caution (compare e.g. SAP102 PDZ1 and PDZ2 with the C terminus of NR2b (17, 35) and the PSD-95 and SAP102 PDZ2 domains with a range of peptides (17), as compared with surface plasmon resonance studies on the very similar SAP97/hDlg PDZ2 (36)). In another study (37), the affinity between PTP-BL PDZ2 and a 19-residue peptide ending with YLVTSV was determined as 8.1 nM using surface plasmon resonance. The on-rate constant was $1.6 \times 10^8 M^{-1} s^{-1}$, and the off-rate constant 0.0013 s$^{-1}$. Thus both the on-rate and off-rate constants from surface plasmon resonance studies on PDZ-peptide interactions were lower than those obtained in the present study. On-rate constants may be different because of differences in the total charges of the interacting molecules and errors in the concentrations of the varied species, but the basis of the $>1000$-fold difference for the off-rate constants must be sought elsewhere. Are the higher off-rate constants determined in this study reasonable from a biological point of view? For regulatory interactions such as those between PDZ domains and their target proteins the answer may well be yes. Off-rate constants in the 5–10 s$^{-1}$ range combine a decent affinity (low $\mu M$) with a fast response to changing cellular conditions, which appears desirable.

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