Binding of Nisin Z to Bilayer Vesicles As Determined with Isothermal Titration Calorimetry†

E. Breukink,‡ P. Ganz,§ B. de Kruijff,‡ and J. Seelig*,§

Department of Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Utrecht, The Netherlands, and Department of Biophysical Chemistry, Biozentrum of the University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland

Received April 21, 2000; Revised Manuscript Received June 16, 2000

ABSTRACT: Nisin Z, a 34-residue lantibiotic, is secreted by some lactic acid bacteria and exerts its antibacterial activity against various Gram-positive bacteria by permeabilizing the cell membrane. It is a cationic amphiphilic peptide with several unusual dehydro residues and thioether-bridged lanthionines. Isothermal titration calorimetry was used to provide a quantitative thermodynamic description for nisin Z adsorption to and penetration into negatively charged and neutral lipid bilayers. The binding of the cationic peptide (electric charge $z \sim 3.8$) to anionic membranes was found to be dominated by electrostatic forces which could be described with the Gouy−Chapman theory. For biologically relevant conditions with a membrane surface potential of −40 mV, the peptide concentration near the membrane surface increases by about 2−3 orders of magnitude compared to the bulk concentration. The binding step proper, i.e., the transition from the lipid−water interface into the membrane, is almost exclusively driven by the high surface concentration. Binding can be described by a partition equilibrium of the form $X_b = KC_M = KC_{p,f} \exp(-z_p\psi_0 F_0/RT)$, where C_M is the peptide surface concentration, $C_{p,f}$ the bulk concentration, and ψ_0 the membrane surface potential. The intrinsic partition coefficient ($K = 1.8\ M^{-1}$) is remarkably small, indicating a correspondingly small hydrophobic energy contribution to the binding process. The electrostatic model was confirmed with nisin Z mutants in which valine-32 was replaced with either lysine (V32K) or glutamate (V32E), increasing or decreasing the electric charge by 1 unit. The extent of peptide binding increased for V32K and decreased for V32E as predicted by the electrostatic theory. In contrast, electrostatic effects were almost negligible for the binding of nisin Z to neutral membranes. However, the binding isotherms were characterized by a distinctly larger intrinsic binding constant K_0 of ∼540 M^{-1} and an enhanced hydrophobic free energy of binding. The binding of nisin Z to sonicated lipid vesicles is exothermic with a $\Delta H°$ of ca. −9 and −3.4 kcal/mol for charged and neutral membranes, respectively.

Nisin, a 34-residue peptide, is an antimicrobial, cationic peptide with intramolecular thioether bridges which has found application as a food preservative (for reviews, see refs *1*−*5*). The peptide renders the bacterial cytoplasmic membrane permeable to ions, amino acids, and ATP and interacts with most membrane lipids in a nonspecific manner (*6*). Its affinity for lipid membranes is sensitive to the lipid composition and increases for negatively charged lipids (*6*−*10*). The presence of a transmembrane potential of sufficient height (inside negative) increases the level of perturbation of the membrane permeability induced by nisin (*11*, *12*). At sufficiently high concentrations, nisin affects the overall organization of lipid bilayers by promoting the inverse hexagonal phase (H_{II}) structure (*13*).

The binding of nisin to lipid membranes has been studied with different techniques such as the monolayer trough (*7*), fluorescence spectroscopy (*12*, *14*), or equilibrium dialysis (*9*). Qualitative or semiquantitative data for the binding process were obtained, but detailed quantitative information about the binding process is still missing. The investigation presented here has two purposes. First, the binding/adsorption of nisin Z to neutral and charged lipid bilayers is assessed with high-sensitivity isothermal titration calorimetry (ITC),[1] yielding both the binding isotherm and the binding enthalpy. Second, we analyze the experimental binding/adsorption isotherm in terms of a combined electrostatic adsorption−hydrophobic partition model. The interaction of a peptide with a lipid membrane can be purely electrostatic as, for example, with polylysines (*15*, *16*) or purely hydrophobic as for cyclosporin A (*17*, *18*), but for most peptides, an intermediate situation will be encountered. Charged peptides will be attracted electrostatically to the membrane surface

† Supported by Swiss National Science Foundation Grant 31-42058.94 and The Netherlands Foundation of Chemical Sciences and Applied Sciences (CW/STW) with financial aid from The Netherlands Foundation of Scientific Research (NWO).

* To whom correspondence should be addressed: Department of Biophysical Chemistry, Biozentrum of the University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland. Telephone: ++41-61-267 2190. Fax: ++41-61-267 2189. E-mail: joachim.seelig@unibas.ch.

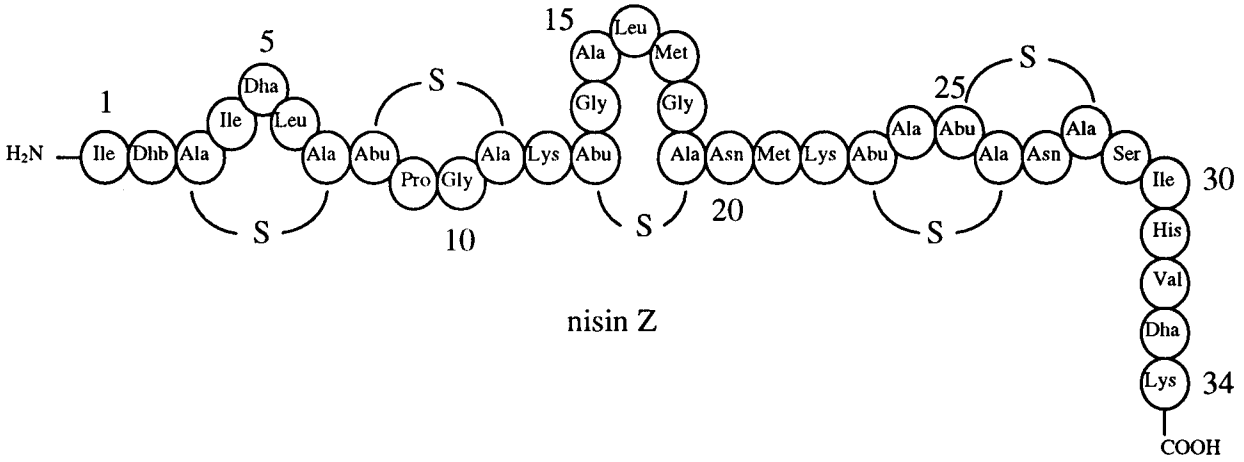
‡ Utrecht University.

§ Biozentrum of the University.

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-*rac*-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-*rac*-phosphoglycerol; ITC, isothermal titration calorimetry; SUV, sonicated unilamellar vesicles.

10.1021/bi000915q CCC: $19.00
Published on Web 07/27/2000

Chart 1



nisin Z

and will then penetrate to some extent into the hydrophobic part of the membrane. Under these circumstances, the quantitative analysis must be based on the Gouy−Chapman theory which has been applied successfully, e.g., for somatostatin derivatives with varying electric charge ($z = 1-3$) (*19*−*21*), for magainin antibiotics ($z = 3-4.8$) (*22*−*25*), and for several other peptides investigated in particular by McLaughlin and co-workers (*15*, *26*−*29*). When the same approach is applied to the nisin binding isotherms, it is possible to separate electrostatic from hydrophobic contributions of the binding process, to determine the intrinsic binding constant, and to determine the free energy and entropy of binding.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-*rac*-phosphoglycerol (POPG), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-*sn*-glycero-3-*rac*-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). All other chemicals were analytical or reagent grade. Buffer was prepared from 18 MΩ water obtained from a NANOpure A filtration system.

All measurements were carried out with nisin Z which was obtained by batch fermentation and purified as described previously (*30*). The nisin Z mutants V32K and V32E were obtained by site-directed mutagenesis, purified, and characterized (*31*). The nisin Z sequence is shown in Chart 1 (*32*).

Preparation of Sonicated Unilamellar Vesicles (SUV). A defined amount of lipid in chloroform was first dried under a nitrogen stream. To remove traces of ethanol which is frequently used to stabilize chloroform, the lipid was dissolved in dichloromethane and then again dried under nitrogen and subsequently overnight under high vacuum. Typically, 2−3 mL of buffer was added to the lipid and the dispersion was extensively vortexed. If not otherwise stated, all experiments were performed in 20 mM MES, 100 mM NaCl buffer (pH 6.5). A few experiments were also carried out with 20 mM phosphate buffer with 100 mM NaCl (pH 6.44). For preparation of small unilamellar vesicles (SUV), the lipid dispersion was sonified (at 4 °C, in ice and water) using a titanium tip ultrasonicator until the solution became transparent. Titanium debris was removed by centrifugation (Eppendorf tabletop centrifuge, 10 min at 14 000 rpm). The lipid concentration was calculated on the basis of the weight of the dried lipid.

High-Sensitivity Isothermal Titration Calorimetry. Isothermal titration calorimetry was performed using a MicroCal Omega high-sensitivity titration calorimeter (Microcal, Norhampton, MA) (*33*). Solutions were degassed under vacuum prior to use. The calorimeter was calibrated electrically. The heats of dilution were determined in control experiments by injecting either peptide solution or lipid suspension into buffer. The heats of dilution were subtracted from the heats determined in the corresponding peptide−lipid binding experiments.

Leakage Experiments. Carboxyfluorescein-loaded DOPC and DOPG vesicles were generated as described previously (*9*). The nisin (or magainin)-induced leakage of carboxyfluorescein from the vesicles was monitored by measuring the increase in fluorescence intensity at 515 nm (excitation at 492 nm) on a SPF 500 C spectrophotometer (SLM Instruments Inc.) at 20 °C in 50 mM MES (pH 6.0) and 100 mM K_2SO_4.

RESULTS

Charged Membranes. High-sensitivity isothermal titration calorimetry was employed to study the binding of nisin Z to phospholipid vesicles. In a first type of experiment, the calorimeter cell contained lipid vesicles and small amounts of a nisin solution were injected. Under the conditions of the experiment, the lipid was in large excess over the injected peptide, leading to an almost complete binding of the peptide. The peptide "binding" (which, more correctly, is a physical adsorption) was found to be exothermic with a reaction enthalpy $\Delta H°$ of no more than −7 kcal/mol.

In a second type of experiment, the peptide solution was filled into the calorimeter cell and unilamellar lipid vesicles were injected. Figure 1A shows a titration sequence where 10 μL aliquots of a phospholipid vesicle suspension [DOPC/DOPG (3/1 molar ratio); total lipid concentration $C_L°$ of 35.4 mM] were injected into the reaction cell ($V_{cell} = 1.3450$ mL) containing a 20 μM nisin Z solution (both solutions in the same buffer, at 28 °C). Each injection produced an exothermic heat of reaction, h_i, which decreased in magnitude with consecutive injections. As a control, buffer without lipid was injected into the peptide solution under the same conditions. The heat of dilution h_{dil} was ∼0.5 μcal per injection. The

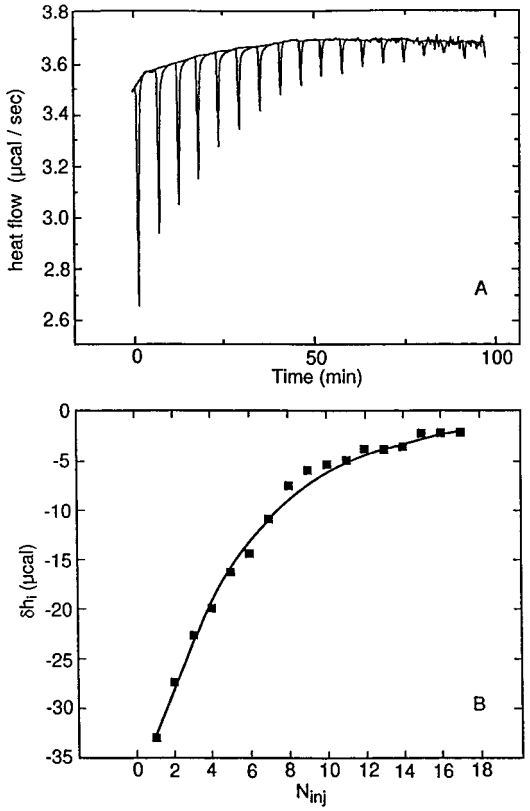


FIGURE 1: Titration calorimetry of nisin Z with DOPC/DOPG (3/1 molar ratio) sonicated unilamellar phospholipid vesicles. (A) Nisin Z ($C_p^0 = 20$ μM) dissolved in buffer [0.1 M NaCl and 20 mM MES (pH 6.5)] titrated with phospholipid vesicles ($C_p^0 = 35.4$ mM) suspended in the same buffer. Each titration peak corresponds to the injection of 10 μL of lipid vesicles. The temperature was 28 °C. (B) Heats of injection, δh_i, as a function of the injection number, N_{inj}. The solid line is the best theoretical fit to the experimental data using a surface partition equilibrium with a K of 1.8 M^{-1} and a ΔH of -9.1 kcal/mol and calculating the increased nisin Z concentration at the membrane surface by using the Gouy−Chapman theory. The average charge of the peptide $\langle z \rangle$ was ≈3.8.

heat of reaction ($\delta h_i = h_i - h_{dil}$) as a function of the injection number (N_{inj}) is shown in Figure 1B. The reaction is complete after about 15 injections, and the reaction enthalpy, ΔH, can then be evaluated with the equation $\Delta H = \Sigma_i \delta h_i / (C_{pep}^0 V_{cell}) = -8.5$ kcal/mol, where C_p^0 is the total peptide concentration in the calorimeter cell. Titration experiments were performed at nisin Z concentrations of up to ~60 μM, and the results are summarized in Table 1. The different titration curves with mixed PC/PG (3/1) membranes led to an average reaction enthalpy ΔH of -9.3 ± 0.6 kcal/mol in MES buffer.

Nisin Z binding to negatively charged membranes should be dominated by electrostatic interactions since nisin Z carries a net charge $\langle z \rangle$ of ~3.3 at pH 6.5. To test this hypothesis, binding studies were performed with V32K and V32E, where valine 32 was replaced with lysine and glutamate, respectively (*31*), changing the electric charge Δz by ± 1. The calorimetric titration pattern obtained for V32K is shown in Figure 2A. The binding reaction is complete after about 10 lipid injections (compared to about 15 injections for wild-type nisin Z), indicating a stronger binding of this peptide. The opposite result is obtained for the less charged V32E (data not shown). The titration peaks, δh_i, decrease less steeply, and the binding is not yet finished after 25 injections. The appearance of the titration patterns qualitatively confirms the dominant role of electrostatic attractions. A quantitative analysis of the binding electrostatics in terms of the Gouy−Chapman theory will be given below. The reaction enthalpy of V32K is -1.9 kcal/mol, and that of V32E is -7.0 kcal/mol.

Table 1: Thermodynamic Parameters for the Binding of Nisin Z to DOPC/DOPG Unilamellar Vesicles with Varying Compositions at 28 °C[a]

compound	PC/PG (%/%)	C_p^0 (μM)	C_L^0 (mM)	$\Delta H°$ (kcal/mol)	K (M^{-1})	z_{eff}[b]
nisin Z	100/0	60	21.6	−3.3	500[c]	0
		64.1	31.0	−3.4	500[c]	0
	75/25	10	40.9[d]	−7.8	1.8	3.8
		20	35.4	−8.5	1.8	3.8
		30	18.15	−9.9	1.8	3.8
		40	18.2	−9.7	1.8	3.8
		50	35.8	−12.2	1.8	3.8
		60	18.1	−8.6	1.8	3.8
		60	37.0	−8.6	1.8	3.8
		61	50.14[e]	−7.8	1.8	3.8
	50/50	2.5−40	~20	-6.5 ± 0.5	ND[f]	
V32K	75/25	20	36.7	−1.9	1.0	4.8
V32E	75/25	20	17.16	−7.0	5.0	2.8

[a] Buffer composed of 20 mM MES and 100 mM NaCl (pH 6.5). [b] Electric charge of the peptide as seen by the membrane surface. [c] Calculated with the assumption that nisin Z binds to the vesicle outside only (60% of the total lipid). [d] POPC/POPG (3/1 molar ratio) lipid composition; buffer composed of 20 mM phosphate and 100 mM NaCl (pH 6.44). [e] POPC/POPG (3/1) lipid composition; buffer composed of 50 mM phosphate and 100 mM NaCl (pH 6.43). [f] ND, not determined.

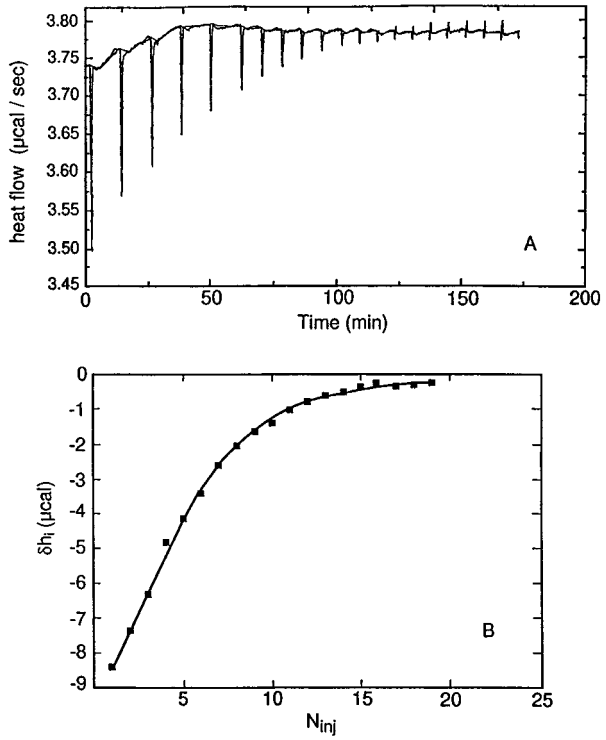


FIGURE 2: Influence of peptide charge. Titration calorimetry of V32K nisin Z. The replacement of valine 32 with lysins increases the average peptide charge $\langle z \rangle$ to 4.8. (A) Titration of V32K nisin Z ($C_p^0 = 20$ μM) with DOPC/DOPG vesicles ($C_p^0 = 37.4$ mM), both in the same buffer [0.1 M NaCl and 20 mM MES (pH 6.5)], with 10 μL injections of lipid. The temperature was 28 °C. (B) Variation of the heat of reaction as a function of injection number, N_{inj}. The solid line corresponds to the best theoretical fit calculated with a K of 1.0 M^{-1} and a ΔH of -1.86 kcal/mol.

As an additional control, nisin Z binding experiments were performed at an increased salt concentration of 0.1 M NaCl and 50 mM phosphate (pH 6.5) [reference measurements with 0.1 M NaCl and 20 mM phosphate (pH 6.5)]. The electrostatic Gouy−Chapman theory predicts a ∼20% reduction in the level of nisin Z binding which was indeed observed experimentally (data not shown).

Electrostatic interactions can be enhanced by employing lipid vesicles with a higher negative surface charge density. Previous investigations have relied on either pure DOPG vesicles or 1/1 DOPG/DOPC mixtures to increase the level of peptide binding (*9*, *12*, *14*, *34*). We have also performed experiments with 1/1 DOPC/DOPG vesicles in the nisin concentration range of 2.5−40 μM. As expected, the titration isotherms are steeper than observed for 3/1 DOPC/DOPG vesicles, but the reaction enthalpy is less exothermic with a $\Delta H°$ of -6.5 ± 1 kcal/mol. Attempts to describe the binding isotherm with the Gouy−Chapman theory and the surface partition model were unsuccessful. The experimental binding isotherm was always steeper than predicted by theory. Additional effects, such as lipid clustering or counterion condensation, may come into play, but were not further investigated.

Neutral Membranes. The adsorption of the cationic peptide to a neutral membrane imparts a positive charge to the bilayer surface, making the approach of further molecules more difficult. The electrostatic interactions are thus repulsive, reducing the extent of peptide binding. This is indeed borne out experimentally. Figure 3A shows the titration of a nisin Z solution ($C_p^0 = 64.1$ μM) with sonicated unilamellar DOPC vesicles ($C_L^0 = 31$ mM). The heats of reaction (Figure 3B) are distinctly smaller in magnitude and vary less steeply than those observed for charged DOPC/DOPG vesicles under similar conditions. However, the non-zero heats of reaction provide evidence for the binding of nisin Z also to neutral membranes. (A titration of a 20 μM nisin Z solution with DOPC vesicles gave correspondingly smaller exothermic reactions.) The binding enthalpy is at least −3.4 kcal/mol but could be as large as −8 kcal/mol (cf. below).

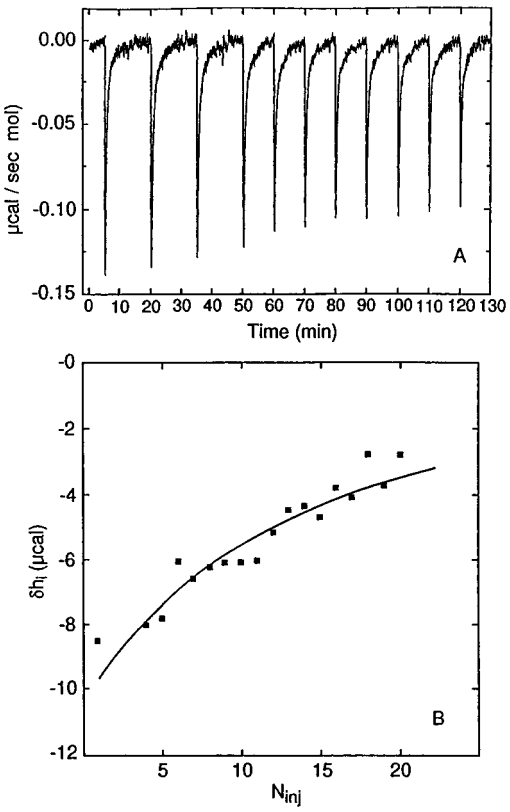


FIGURE 3: Nisin Z binding to neutral membranes. (A) Titration of a 64.1 μM nisin Z solution with a dispersion of neutral DOPC vesicles ($C_L^0 = 31.0$ mM), both in buffer [0.1 M NaCl and 20 mM MES (pH 6.5)], with 5 μL injections of lipid. The temperature was 28 °C. (B) Variation of the heat of reaction as a function of lipid injection. The solid line is the best theoretical fit, calculated with a $\langle z \rangle$ of 0.96, a K of 325 M^{-1}, and a ΔH of −3.4 kcal/mol.

DISCUSSION

Theoretical Analysis. The titration experiment can be used to derive the binding isotherm, $X_b = f(C_{p,f})$, where X_b is the molar amount of bound peptide per mole of lipid (accessible for binding) and $C_{p,f}$ is the equilibrium concentration of peptide in bulk solution (*17*). Let us denote with $\Theta_{p,b}^i$ the mole fraction of peptide bound to the lipid vesicles after i lipid injections. It can be evaluated from the experimental δh_i:

$$\Theta_{p,b}^{\ i} = \sum_{k=1}^{i} \delta h_i / (\Delta H V_{cell} C_p^{\ 0}) \qquad (1)$$

The free peptide concentrations, $C_{p,f}^i$, are then given by

$$C_{p,f}^{\ i} = C_p^{\ 0}(1 - \Theta_{p,b}^{\ i}) f_{dil}^{\ i} \qquad (2)$$

The dilution factor takes into account the increase in reaction volume due to lipid vesicle injection and is defined as

$$f_{dil}^{\ i} = \left(\frac{V_{cell}}{V_{cell} + V_{inj}}\right) \approx \frac{V_{cell}}{V_{cell} + iV_{inj}} \qquad (3)$$

where V_{inj} is the injected volume per reaction step ($V_{inj}/V_{cell} \ll 1$). The extent of peptide binding, X_b^i, is defined as the molar ratio of bound peptide, $n_{pep,b}^i$, to accessible lipid, n_L^i. The lipid content increases with each injection according to $iV_{inj}C_L^0$ and X_b^i is thus given

$$X_b^{\ i} = \Theta_{p,b}^{\ i} \frac{C_{pep}^{\ 0} V_{cell}}{iV_{inj} C_L^{\ 0}} \qquad (4)$$

C_L^0 is the concentration of either the total lipid (if nisin Z can rapidly translocate from the membrane outside to the inner half-layer) or, in the absence of translocation, the lipid in the outer half-layer only ($0.6C_L^0$). A plot of X_b^i versus $C_{p,f}^i$ yields the desired binding isotherm, i.e., $X_b = f(C_{p,f})$.

Next, the binding isotherm is analyzed in terms of an electrostatic model described previously for the magainin 2 antibiotics (*22*, *35*). The model first calculates the concentration of the peptide at the membrane surface, C_M. The latter is distinctly larger than the bulk equilibrium concentration, $C_{p,f}$, because the membrane and nisin Z are oppositely charged and the peptide is electrostatically attracted to the membrane surface. The surface concentration is given by the Boltzmann equation:

$$C_M = C_{p,f} e^{-(z_p F_0 \psi_0)/RT} \qquad (5)$$

where z_p is the signed charge of the peptide, F_0 is the Faraday constant, and RT is the thermal energy. The membrane surface potential, ψ_0, must be calculated with the Gouy−Chapman theory (*19*).

In a second step, the surface concentration C_M is used to calculate the amount of bound peptide, X_b, according to the surface partition model

$$X_b = KC_M \quad (6)$$

where K is the surface partition coefficient. At this stage, electrostatic concentration differences have been eliminated and K reflects exclusively hydrophobic and other nonelectrostatic energies of binding.

Binding Isotherms of Negatively Charged Membranes. The theory outlined above was used to fit the titration curves $\delta h_i = f(N_{inj})$ and to find the optimum parameters for K and ΔH. Figure 1B (solid line) shows the result of such an analysis calculated with a partition constant K of 1.8 M^{-1} and a reaction enthalpy ΔH of −9.1 kcal/mol, assuming a rapid translocation of nisin Z across the membrane. Na^+ binding to POPG was taken into account with a binding constant K_{Na^+} of 0.6 M^{-1} (*36*−*38*). The negative charge of the membrane generates a surface potential ψ_0 of ca. −40 mV and reduces the pH from pH 6.5 in bulk solution to pH 5.8 near the membrane surface. The pK of the N-terminal NH_2 group was assumed to be 7.2 and that of His-31 6.5 (*39*). The peptide charge z_{eff} changes from 3.34 at pH 6.5 in solution to ∼3.8 at the membrane surface at pH 5.8. As a consequence of the large surface potential, the concentration of the peptide, C_M, above the plane of binding is found to be larger by a factor of 200−300 than the bulk concentration, $C_{p,f}$. The corresponding analysis was also performed for V32K ($z \approx 4.8$). Figure 2B again demonstrates an excellent agreement between theory and experiment using a K of 1.0 M^{-1} and a ΔH of −1.86 kcal/mol. The corresponding analysis for V32E ($z \approx 2.8$) yields a K of 5 M^{-1} and a $\Delta H°$ of −7 kcal/mol.

Knowledge of the binding constant K allows the calculation of the binding isotherm. Figure 4 shows two different representations of the nisin Z binding isotherms for DOPC/DOPG (3/1 molar ratio) membranes (in buffer at pH 6.5). In Figure 4A, the level of bound nisin, X_b, is plotted against the nisin Z surface concentration, C_M. The solid line is the theoretical prediction, and the symbols represent the experimental data derived from Figure 1. C_M is not experimentally accessible but must be evaluated using the model described above. The figure confirms the linear relationship between the amount of bound nisin Z and the peptide surface concentration, C_M. Figure 4B shows the same experimental data as a function of the peptide equilibrium concentration, $C_{p,f}$, i.e., the conventional binding isotherm. The binding isotherm is nonlinear, bending toward the x-axis as the membrane surface charge is gradually compensated at higher nisin Z concentrations. The solid line represents again the prediction of the theory.

For each point of the binding isotherm of Figure 4B, an apparent binding constant K_{app} ($=X_b/C_{p,f}$) can be defined. K_{app} is obviously not constant but varies with peptide concentration. For the concentration range shown in Figure 4B, K_{app} varies between ∼1800 M^{-1} near the origin to 550 M^{-1} at 15 μM nisin Z. Nisin Z binding to negatively charged membranes has been investigated previously with different tryptophan mutants taking advantage of a change in fluorescence intensity upon binding (*14*). Electrostatic effects were not considered. The apparent binding constant for 1/1 DOPC/DOPG vesicles was ∼10^4 M^{-1} at a nisin concentration of 1 μM, in broad agreement with the analysis presented here.

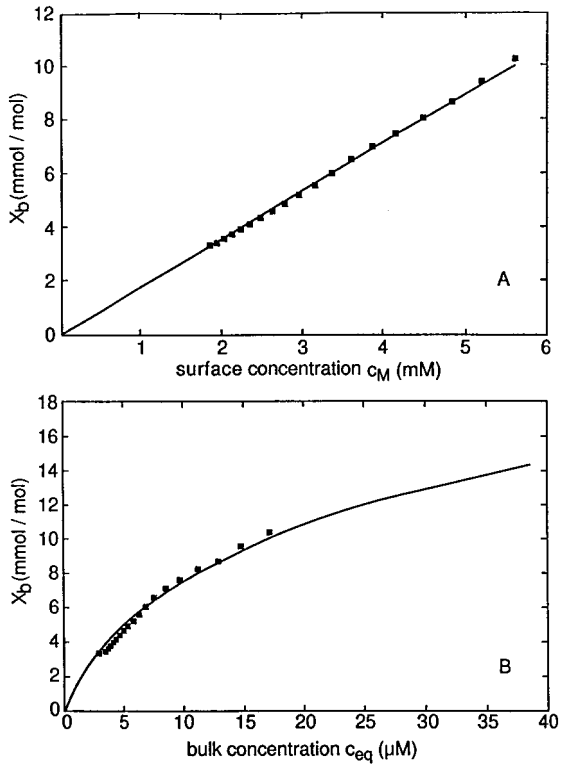


FIGURE 4: Nisin Z binding isotherm for DOPC/DOPG (3/1 molar ratio) membranes. The binding isotherm was calculated with a nisin Z binding constant K of 1.8 M^{-1}. (A) Extent of binding, X_b, as a function of the surface concentration, C_M. (B) Extent of binding, X_b, as a function of the equilibrium concentration, C_{eq}. Note the different scales and units of the two abscissae. The surface concentration, C_M, is about 2 orders of magnitude larger than the equilibrium concentration, C_{eq}. The solid symbols represent the experimental results depicted in Figure 1. The surface potential decreases from about −45 mV at zero peptide concentration to about −38 mV when $C_{p,f} = 20$ μM. Na^+ binding to POPC was taken into account with a binding constant K of 0.6 M^{-1}.

Binding Isotherms of Neutral Membranes. The binding of nisin Z to neutral membranes exhibits a rather flat titration curve (small changes in δh_i between consecutive lipid injections) which excludes strong electrostatic effects. Unexpectedly, the titration isotherms are well-described by a simple partition equilibrium $X_b = KC_{p,f}$ without electrostatic effects ($C_{p,f}$ is the bulk peptide concentration). In this simplified model, only K and $\Delta H°$ are free parameters. If it is assumed that nisin Z binds to the vesicle outside only, K and $\Delta H°$ are determined to be 500 M^{-1} and −3.4 kcal/mol, respectively. As the electric charge is switched on, K increases since repulsive interactions come into play. If a hypothetical peptide charge z_{eff} of 1 is assumed, the electrostatic interactions are still small and K increases modestly to 540 M^{-1}. Again, an almost perfect description of the experimental data is possible. However, if the same calculation is repeated for the true peptide charge of 3.8, the fit between experimental data and theory is poor. No binding constant is found which describes the data over the

whole concentration range. From an empirical point of view, it must be concluded that only a partition model with no or little electrostatic interaction ($z = 1$) provides a satisfying description of the experimentally available data. Fluorescence studies with nisin Z tryptophan mutants and neutral membranes revealed only minor intensity changes (*14*). Very low concentrations of nisin (1 μM) and lipid (100 μM) were used. On the basis of the above binding constant, it can be estimated that no more than 5% of the total nisin Z was bound in these earlier experiments and thus escaped detection against the large background of free nisin.

The differences between the nisin Z binding isotherms for neutral and charged membranes suggest two modes of interaction. For negatively charged membranes, nisin Z appears to maximize its nonspecific electrostatic interactions at the expense of hydrophobic interactions (low *K*); in the presence of neutral lipids, the hydrophobic interaction becomes dominant (large *K*) and the electric charges remain some distance from the membrane (cf. below).

Thermodynamic Aspects of Nisin Z Binding. Knowledge of the intrinsic binding constant allows the calculation of the free energy of binding according to the equation $\Delta G_h^\circ = -RT \ln(55.5K)$. The factor 55.5 corresponds to the molar concentration of water and corrects for the fact that the peptide concentration in solution is not given as its mole fraction but in moles per liter. The hydrophobic free energy contributions (ΔG_h°) are -2.8 and -6.1 kcal/mol for charged and neutral membranes, respectively. The electrostatic term contributes an additional -4.1 kcal/mol in charged membranes. The corresponding binding enthalpies ΔH° are -9.3 and -3.4 kcal/mol. As a first conclusion, it follows that the binding process is driven predominantly or even exclusively by the reaction enthalpy.

Compared to those of other amphipathic peptides such as the magainin antibiotics, the binding enthalpies of nisin Z and its analogues are distinctly less exothermic even though nisin Z is the longer peptide. This difference can be explained by the fact that magainin peptides undergo an exothermic coil → α-helix transition at the membrane surface which contributes about -10 kcal/mol to the binding enthalpy (*23*). No such conformational transition appears to be possible for nisin Z due to the presence of several lanthionine rings.

Finally, it should be noted that the pH near the surface of the negatively charged membrane is lower than that in bulk solution, leading to a protonation of the N-terminal amino group and the His-31 side chain as the peptide approaches the membrane. The protonation enthalpy ΔH_{prot} can be estimated to be -2.1 kcal/mol (*19*).

Membrane Permeability and Nisin Translocation. The biological activity of nisin Z results from a permeabilization of bacterial membranes, causing an efflux of the cytoplasmatic content. The present analysis provides evidence that membrane leakiness parallels the extent of peptide binding. This is illustrated in Figure 5A, where nisin Z and magainin 2 are added to dye-loaded lipid vesicles (composed of either DOPC or DOPG) and the dye efflux is measured as a function of the peptide concentration. Nisin Z is seen to be much more efficient for DOPG than for DOPC vesicles (*9*). The binding isotherms, calculated for nisin Z binding to pure DOPC and mixed DOPC/DOPG (3/1) membranes, are displayed in Figure 5B. A semiquantitative agreement between the two types of experiments is obtained. The dashed line in Figure 5B represents the binding isotherm for nisin Z binding to neutral membranes, calculated with a *K* of 540 M^{-1} and a *z* of ≈1, and the solid line is the binding isotherm of nisin Z binding to charged membranes (3/1 PC/PG) with a *K* of 1.8 M^{-1} and a *z* of ≈3.8. The extent of binding is distinctly larger for charged than for neutral membranes since the electrostatic attraction to the anionic membrane more than compensates for the larger hydrophobic binding to neutral membranes.

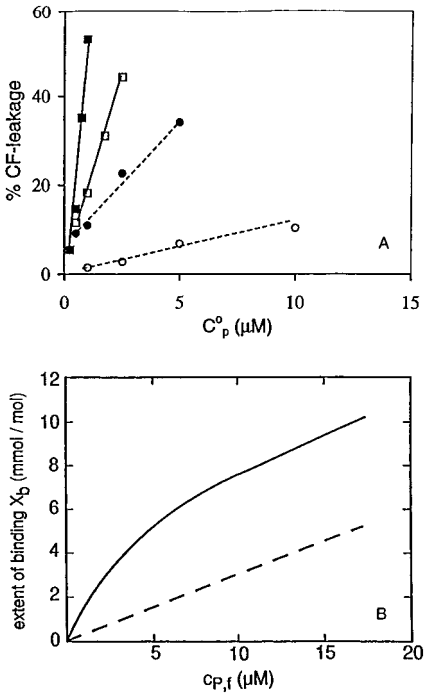


FIGURE 5: (A) Carboxyfluorescein efflux from DOPC or DOPG vesicles as a function of the nisin Z or magainin 2 amide concentration: (■) M2a binding to DOPG vesicles, (□) nisin Z binding to DOPG, (●) M2a/DOPC vesicles, and (○) nisin Z with DOPC. (B) Comparison of nisin Z binding to charged and neutral lipid bilayers. The theoretical binding isotherms were calculated with the parameters found to yield the optimum fit to the experimental data. The solid line is for the charged membrane (3/1 PC/PG) using a *K* of 1.8 M^{-1} and a z_{eff} of ≈3.8. The dashed line is for the neutral membrane (PC) using a *K* of 325 M^{-1} and a z_{eff} of 0.96.

Figure 5A further demonstrates that, for a given membrane system, magainin 2 amide causes a larger dye efflux than nisin Z. While both peptides have about the same electric charge, the intrinsic binding constant of magainin 2 amide is 50 M^{-1} for 3/1 POPC/POPG membranes and 2000 M^{-1} for POPC membranes at 30 °C (*22*). Under equal electrostatic conditions, the enhanced hydrophobic binding constant of magainin 2 amide makes binding of this peptide more favorable than binding of nisin Z. At a free peptide concentration $C_{p,f}$ of 1 μM, the extent of binding is 12 mmol of peptide/mol of lipid for magainin 2 amide but only 1 mmol/mol for nisin Z. The increase in membrane leakiness as induced by magainin 2 amide (Figure 5A) can thus be explained by the larger intrinsic (hydrophobic) binding constant of this peptide.

Membrane leakiness can be induced by either a perturbation of the lipid packing (*13*) or the formation of peptide pores. Both possibilities are discussed in the literature, but a decision between the two alternatives is not possible at present. Peptide pores require a transmembrane orientation

of the peptides that are involved. Experimental evidence for a translocation of nisin Z across negatively charged membranes comes from trypsin digestion experiments in which trypsin was enclosed in the lumen of lipid vesicles (*40*). The evaluation of the nisin Z titration isotherms obtained with charged lipid vesicles was therefore based on 100% lipid accessibility. For neutral membranes, only an asymmetric binding was assumed.

The results presented here on both neutral and charged membranes are in qualitative agreement with surface pressure studies on nisin Z penetration into lipid monolayers with different compositions (*7*). Nisin Z proved to have a high penetration power for anionic lipids and a low penetration power for neutral lipids. At a monolayer surface pressure π of 25 mN/m [i.e., the bilayer $\rightleftarrows$ monolayer equivalence pressure of sonicated vesicles (*41*−*43*)], the increase in monolayer surface pressure, $\Delta\pi$, was ∼6 mN/m for the DOPC monolayer but ∼15 mN/m for pure DOPG monolayers (∼0.5 μM nisin Z). Since the increase in surface pressure is caused by an intercalation of nisin Z between the lipids, the monolayer studies are consistent with the ∼3-fold larger X_b value for charged PC/PG membranes compared to that for neutral PC membranes (Figure 5) at low nisin Z concentrations. Likewise, nisin Z binding to lipid bilayers was investigated previously with a centrifugation assay (*9*), with nonequilibrium gel chromatography (*9*), and with fluorescence spectroscopy (*14*), and the level of binding was found to be distinctly larger for charged membranes than for neutral membranes.

Binding Model. The NMR analysis of nisin Z in solution and in a micellar environment demonstrates that the lanthionine-containing ring systems are well-constrained while the structure of the linear peptide is less defined, leading to a rather flexible molecule (*44*). The presence of five ring systems excludes the possibility of a coil → helix transition at the membrane surface as observed for other amphipathic peptides such as melittin or magainin. Electric charges are found at residues 1 (amino terminus), 12 (Lys), 22 (Lys), 31 (His), and 34 (Lys). On the basis of the NMR analysis, both the N-terminal and C-terminal halves of the molecule have been described as β-structured and amphipathic (*44*). The depth of penetration of nisin Z into lipid bilayers was investigated with nisin Z mutants containing tryptophan residues at different positions in the molecule (*14*) and by Monte Carlo simulations (*45*). Both studies orient the nisin Z molecule with its long axis parallel to the membrane surface as evidenced, for example, by the only modest blue shift (5−12 mm) of the tryptophan emission maximum, indicating a tryptophan location not in the membrane interior but closer to the lipid−water interface.

Thermodynamic measurements are not particularly useful for differentiating between molecular models. Nevertheless, this study provides a number of results which must be taken into account when placing nisin Z into the lipid membrane. First, the interaction of nisin with charged membranes (25% PG) is essentially electrostatic, accounting for about 60% of the total free energy of binding. Second, nisin Z binds significantly to neutral membranes, and the binding energy under these conditions is exclusively hydrophobic. Thus, the orientation and penetration depth of nisin Z cannot be the same for the two types of membranes. A similar result was reported for magainin 2 amide where the intrinsic binding constant was ∼50 M^{-1} for charged membranes (*23*, *35*) and increased to ∼2000 M^{-1} for neutral membranes (*22*). Third, the binding isotherms for charged membranes are best described by assuming a rapid translocation of nisin Z across the membrane. Even if nisin Z is mainly located at the membrane surface, transitory steps (pore formation and lipid perturbation) are required to allow the passage across the membrane interior.

In conclusion, nisin Z binding to lipid membranes can be described in terms of a classical partition model for both neutral and charged membranes. The peptide shows a significant affinity for neutral membranes but binds even better to negatively charged membranes because of nonspecific electrostatic attraction. The electrostatic forces are well-described by the Gouy−Chapman theory, and the theoretical approach is supported by the results obtained with nisin Z mutants with altered peptide charge. For 3/1 PC/PG membranes, ∼60% of the nisin Z free energy of binding comes from electrostatic interactions. The nisin-induced dye leakage through the membrane parallels the extent of peptide binding. For a given amount of bound peptide, membrane leakage appears to be similar for neutral and charged membranes. A specific binding of nisin Z to phosphatidylglycerol is not supported by the experimental resuls. The adsorption to and partial penetration into the membrane is an exothermic process if sonicated unilamellar vesicles are employed. The reaction enthalpy (and not the entropy) is the driving force for binding.

REFERENCES

1. Jung, G. (1991) *Angew. Chem. 103*, 1067−1084.
2. Bierbaum, G., and Sahl, H. G. (1993) *Zentralbl. Bakteriol. 278*, 1−22.
3. Entian, K. D., and Klein, C. (1993) *Naturwissenschaften 80*, 454−460.
4. Hansen, J. N. (1993) *Annu. Rev. Microbiol. 47*, 535−564.
5. Sahl, H. G., Jack, R. W., and Bierbaum, G. (1995) *Eur. J. Biochem. 230*, 827−853.
6. Kordel, M., Schuller, F., and Sahl, H. G. (1989) *FEBS Lett. 244*, 99−102.
7. Demel, R. A., Peelen, T., Siezen, R. J., De Kruijff, B., and Kuipers, O. P. (1996) *Eur. J. Biochem. 235*, 267−274.
8. Martin, I., Ruysschaert, J. M., Sanders, D., and Giffard, C. J. (1996) *Eur. J. Biochem. 239*, 156−164.
9. Breukink, E., van Kraaij, C., Demel, R. A., Siezen, R. J., Kuipers, O. P., and de Kruijff, B. (1997) *Biochemistry 36*, 6968−6976.
10. el-Jastimi, R., and Lafleur, M. (1997) *Biochim. Biophys. Acta 1324*, 151−158.
11. Sahl, H. G., Ersfeld-Dressen, H., Bierbaum, G., Josten, M., Kordel, M., Reis, M., and Schuller, F. (1987) *Zentralbl. Bakteriol., Mikrobiol. Hyg., Abt. 1, Orig. A 267*, 173−185.
12. Driessen, A. J., van den Hooven, H. W., Kuiper, W., van de Kamp, M., Sahl, H. G., Konings, R. N., and Konings, W. N. (1995) *Biochemistry 34*, 1606−1614.
13. El Jastimi, R., Edwards, K., and Lafleur, M. (1999) *Biophys. J. 77*, 842−852.
14. Breukink, E., van Kraaij, C., van Dalen, A., Demel, R. A., Siezen, R. J., de Kruijff, B., and Kuipers, O. P. (1998) *Biochemistry 37*, 8153−8162.
15. Kim, J., Mosior, M., Chung, L. A., Wu, H., and McLaughlin, S. (1991) *Biophys. J. 60*, 135−148.
16. Montich, G., Scarlata, S., McLaughlin, S., Lehrmann, R., and Seelig, J. (1993) *Biochim. Biophys. Acta 1146*, 17−24.
17. Seelig, J. (1997) *Biochim. Biophys. Acta 1331*, 103−116.
18. Schote, U., Fahr, A., and Seelig, J. (2000) *J. Pharm. Sci.* (submitted for publication).

19. Seelig, J., Nebel, S., Ganz, P., and Bruns, C. (1993) *Biochemistry 32*, 9714−9721.
20. Beschiaschvili, G., and Seelig, J. (1990) *Biochemistry 29*, 52−58.
21. Beschiaschvili, G., and Seelig, J. (1992) *Biochemistry 31*, 10044−10053.
22. Wieprecht, T., Beyermann, M., and Seelig, J. (1999) *Biochemistry 38*, 10377−10387.
23. Wieprecht, T., Apostolov, O., Beyermann, M., and Seelig, J. (1999) *J. Mol. Biol. 294*, 785−794.
24. Wieprecht, T., Apostolov, O., Beyermann, M., and Seelig, J. (2000) *Biochemistry 39*, 442−452.
25. Wieprecht, T., Apostolov, O., and Seelig, J. (2000) *Biophys. Chem.* (in press).
26. Mosior, M., and McLaughlin, S. (1992) *Biochim. Biophys. Acta 1105*, 185−187.
27. Mosior, M., and McLaughlin, S. (1992) *Biochemistry 31*, 1767−1773.
28. Peitzsch, R. M., and McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.
29. Ben-Tal, N., Honig, B., Peitzsch, R. M., Denisov, G., and McLaughlin, S. (1996) *Biophys. J. 71*, 561−575.
30. Kuipers, O. P., Rollema, H. S., Yap, W., Boot, H. J., Siezen, R. J., and Devos, W. M. (1992) *J. Biol. Chem. 267*, 24340−24346.
31. Van Kraaij, C., Breukink, E., Rollema, H. S., Siezen, R. J., Demel, R. A., De Kruijff, B., and Kuipers, O. P. (1997) *Eur. J. Biochem. 247*, 114−120.
32. Piard, J. C., Kuipers, O. P., Rollema, H. S., Desmazeaud, M. J., and de Vos, W. M. (1993) *J. Biol. Chem. 268*, 16361−16368.
33. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem. 179*, 131−137.
34. Giffard, C. J., Dodd, H. M., Horn, N., Ladha, S., Mackie, A. R., Parr, A., Gasson, M. J., and Sanders, D. (1997) *Biochemistry 36*, 3802−3810.
35. Wenk, M. R., and Seelig, J. (1998) *Biochemistry 37*, 3909−3916.
36. Eisenberg, M., Gresalfi, T., Riccio, T., and McLaughlin, S. (1979) *Biochemistry 18*, 5213−5223.
37. Macdonald, P. M., and Seelig, J. (1987) *Biochemistry 26*, 1231−1240.
38. Macdonald, P. M., and Seelig, J. (1987) *Biochemistry 26*, 6292−6298.
39. Martin, B. R. (1964) *Introduction to Biophysical Chemistry*.
40. van Kraaij, C., Breukink, E., Noordermeer, M. A., Demel, R. A., Siezen, R. J., Kuipers, O. P., and de Kruijff, B. (1998) *Biochemistry 37*, 16033−16040.
41. Demel, R. A., Geurts van Kessel, W. S., Zwaal, R. F., Roelofsen, B., and van Deenen, L. L. (1975) *Biochim. Biophys. Acta 406*, 97−107.
42. Schindler, H. (1979) *Biochim. Biophys. Acta 555*, 316−336.
43. Seelig, A. (1987) *Biochim. Biophys. Acta 899*, 196−204.
44. Van Den Hooven, H. W., Doeland, C. C., Van De Kamp, M., Konings, R. N., Hilbers, C. W., and Van De Ven, F. J. (1996) *Eur. J. Biochem. 235*, 382−393.
45. Lins, L., Ducarme, P., Breukink, E., and Brasseur, R. (1999) *Biochim. Biophys. Acta 1420*, 111−120.

BI000915Q