Evidence for Protonation in the Human Neutrophil Formyl Peptide Receptor Binding Pocket†

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ABSTRACT: We have studied the interaction of a family of fluorescent formyl peptides with their receptor using spectrofluorometric and flow cytometric methods. The peptides contained four (CHO-Met-Leu-Phe-Lys-fluorescein), five (CHO-Met-Leu-Phe-Phe-Lys-fluorescein), or six (CHO-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein) amino acids. As observed in earlier studies, the fluorescent peptides containing four and five amino acids were quenched upon binding to the receptor, while the hexapeptide was not. While the degree of quenching of the bound tetrapeptide was largely unchanged, the quenching of the bound pentapeptide decreased with increasing pH over the range of pH 6.5–9.0. Ligand binding studies have shown that the mole fraction of tetrapeptide or pentapeptide bound in kinetic analysis markedly decreased with increasing pH as a consequence of increasing ligand dissociation rate constant. The dependence of the binding parameters for the hexapeptide on pH was much less pronounced. Over a pH range from pH 7.3 to 9.0, the hexapeptide showed little change in binding affinity, while the tetrapeptide and pentapeptide increased in K_d approximately 2.0- and 2.5-fold, respectively. These results indicate that the formyl peptide receptor binding pocket contains at least two microenvironments. The pH sensitivity of the pentapeptide quenching is consistent with a protonating environment, while the pH-independent quenching of the tetrapeptide may reflect aromatic stacking or a hydrophobic microenvironment. The pH-dependent ligand dissociation also suggests that the protonation in the pocket stabilizes ligand binding, which may indicate an alteration in the binding pocket structure. Protonation or hydrogen bonding of the pentapeptide may lead to even further stabilization of that ligand.

Cell surface receptors specific for peptide ligands are found throughout nature. The human neutrophil formyl peptide receptor is linked via a G protein to cell responses essential in the defense against bacterial infection. Amino acid sequences of such signaling receptor proteins suggest that they form a family of related molecules which span the plasma membrane with seven α-helical, transmembrane domains (Strader et al., 1989; Boulay et al., 1990) with the ligand binding pocket formed by a cleft located within these membrane-spanning regions. Traditionally, the interactions between receptor binding pocket and ligand are defined by structure–activity and structure–affinity relationships of families of structurally related ligand molecules. Such studies with the formyl peptide receptor indicate that at least four or five amino acids from the amino terminus of the ligand contribute to its activity (Niedel et al., 1979; Freer et al., 1982) and that the binding pocket must accommodate molecules of at least that dimension.

A different approach is proving to be of great utility in detecting and quantitating formyl peptide receptors on neutrophils at natural abundance (Sklar et al., 1984, 1989). This approach uses the environmental sensitivity of ligand molecules covalently modified by fluorescent probes to examine structural features of the ligand–receptor interaction. Fluorescein (Haugland et al., 1989) and derivatized FITC[1] (Mercola et al., 1972) exhibit decreased fluorescence intensity with decreasing pH, because the protonated forms of the molecules are nonfluorescent. The fluorescence is also quenched by interaction with hydrophobic aromatic residues (e.g., tryptophan) in solution and in protein binding pockets (Watt & Voss, 1977; Herron et al. 1989).

The binding of peptide ligands of different lengths to the formyl peptide receptor is associated with fluorescence quenching. Moreover, the accessibility of the chromophore to the external environment increases with the peptide length. Together, these observations suggest that the binding pocket accommodates no more than six amino acids (Sklar et al., 1990) and that the chromophore resides within the pocket for the shorter ligands. However, little is known about the specific interactions of formyl peptide ligands with the interior of the binding pocket. In this paper, we therefore took advantage of the environmental sensitivity of the FITC-labeled formyl peptide ligands to investigate the basis of the quenching. We report that the formyl peptide receptor binding pocket contains at least two microenvironments: one hydrophobic and the other protonating. From the binding characteristics of these ligands, we suggest that protonation of the receptor acts to stabilize ligand binding, perhaps through a conformational change in the pocket. Environmentally sensitive probes may prove generally useful to define receptor binding pockets in many different systems.

MATERIALS AND METHODS

Neutrophils and Permeabilized Neutrophils. Human neutrophils were prepared by the elutriation method of Tolley et al. (1987). Permeabilization (Sklar et al., 1987) required neutrophils suspended at 25×10^6/mL to be incubated for 25

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[1] Abbreviations: FITC, fluorescein 5′-isothiocyanate (isomer I); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHO, *N*-formyl group of formylated peptides; IBB, intracellular binding buffer; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; D MSO, dimethyl sulfoxide; tBoc, *tert*-butyloxycarbonyl; GTP[S], guanosine 5′-O-(3-thiotriphosphate); I_b/I_f, the ratio of the intensities of receptor bound and free peptide; X_b, the mole fraction of ligand bound in a reaction; RT, room temperature.

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min at 37 °C with 15 μg/mL digitonin in 100 mM KCl, 20 mM NaCl, 1 mM EGTA, and 30 mM Hepes, pH 7.3). The intracellular binding buffer (IBB) was made by supplementing the above buffer with 0.1% BSA, 1 mM PMSF, and 5 mM $MgCl_2$.

Fluorescent Formyl Peptides. The hexapeptide CHO-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein (FITC isomer I) was obtained from Molecular Probes (Eugene, OR). The tetrapeptide CHO-Met-Leu-Phe-Lys-fluorescein was obtained from Peninsula Laboratories (Burlingame, CA). The pentapeptide CHO-Met-Leu-Phe-Phe-Lys-fluorescein was prepared by the rapid mixed anhydride procedure (Muthukumaraswamy & Freer, 1987) as described by Sklar et al. (1989). Peptides were stored as aliquots in DMSO at concentrations ranging from 100 μM to 1 mM or as dry powders.

Miscellaneous Reagents. The polyclonal antibody to fluorescein was prepared (Sklar, 1987) and its high-affinity binding quantitated as previously described (Levison et al., 1975). The formyl peptide antagonist tBoc-Phe-Leu-Phe-Leu-Phe was obtained from Vega Biochemicals (Tucson, AZ). GTP[S] was obtained from Sigma (St. Louis, MO). Fluorescent standard beads were obtained from Flow Cytometry Standards Corp. (Research Triangle Park, NC).

Antibody Binding Method (Sklar et al., 1989). For all fluorescent peptides used, permeabilized cells were suspended at 10^7/mL in IBB (pH 6.5–9.0) and 200-μL aliquots were placed in the stirred cylindrical cuvette of the SLM 8000. The fluorescence was monitored continuously following the addition of fluorescent peptide at a final concentration of 1 nM (Figure 1). Binding was allowed to proceed for the desired period of time (120 s) in the presence or absence of excess blocking peptide (tBoc-Phe-Leu-Phe-Leu-Phe) followed by the addition of antibody to fluorescein (2 μL, ~2500 nM) to the cuvette; the mixture was allowed to react for 120 s. Antibody binds and quenches the free fluorescent peptide within 1 s at a rate constant of $\sim 10^8$ M·s^{-1}. In contrast, antibody recognizes the receptor-bound hexapeptides with a half-time of ~300 s and with a rate constant of $\sim 10^5$ M·s^{-1}. Fluorescein on the shorter peptides is protected in the binding pocket of the cell surface receptor and is entirely inaccessible to antibody to fluorescein (Sklar et al., 1990). For all the peptides, the addition of antibody discriminates the free and bound ligand. Permeabilization is verified by the ability of GTP[S] to reduce the ligand affinity, releasing the bound peptide for quenching by the antibody.

Quenching within the Receptor Binding Pocket. The antibody to fluorescein is used in the evaluation of the relative intensities of the free and bound ligand as well as of the mole fraction of bound ligand. As illustrated for the tetrapeptide at pH 9.0 (Figure 1, top panel), the sample containing excess nonfluorescent ligand traces the curve connected by *a* (free ligand) and *d* (ligand bound to antiobody); samples with receptor binding trace the curves connected by *b* and *c*. Curves for blocked samples were essentially the same as shown for the tetrapeptide. The fluorescence values *a*, *b*, *c*, and *d* can be related to the intensities and mole fractions of the fluorescent bound ligand according to the following scheme (Sklar et al., 1990). I_f is the intensity of free peptide, I_b is the intensity of cell-bound peptide, I_a is the intensity of antibody-bound peptide, and X_b is the mole fraction of cell-bound peptide. Then *a* represents free ligand with intensity I_f, *b* represents a combination of free ligand and quenched bound ligand where the intensity is given by $I_f(1 - X_b) + I_bX_b$; *c* represents a combination of receptor-bound ligand and antibody-bound ligand, the intensity being given by $I_bX_b + I_a(1 - X_b)$, and *d* represents the ligand bound to antibody where the intensity

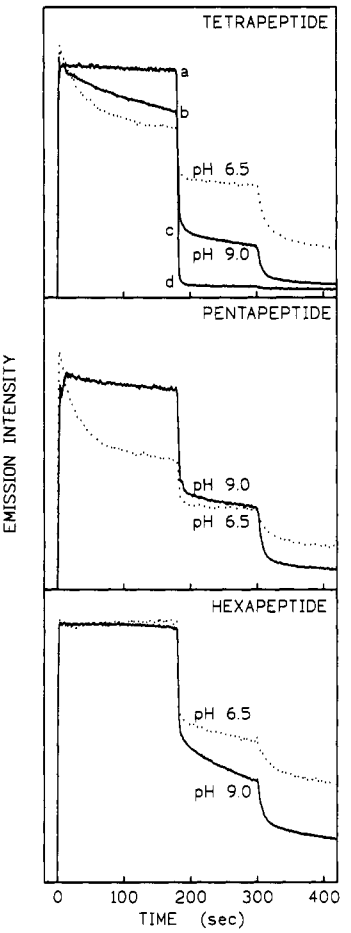

FIGURE 1: Fluorescent peptide binding and dissociation at pH 9.0 and 6.5. Spectrofluorometric binding experiments with either tetrapeptide, pentapeptide, or hexapeptide were performed over a pH range from pH 6.5 to 9.0 as described in Materials and Methods. Peptide is added at time 0, antibody at 180 s to measure binding, and GTP[S] at 300 s to confirm permeabilization. A representative control in which excess nonfluorescent blocking peptide (curve ad) is present is shown in the top panel. Data were normalized to reflect differences in fluorescence emission intensity vs pH.

is I_a. By rearrangement and substitution, the residual fluorescence of the bound ligand I_b/I_f is given by

$$I_b/I_f = [(b - a)(a - d)/a(a - b + c - d)] + 1 \quad (1)$$

and the mole fraction bound, X_b, is given by

$$X_b = 1 - [(b - c)/(a - d)] \quad (2)$$

Residual fluorescence and binding values are plotted vs pH in Figures 2 and 3, respectively, for all ligands. Data collected after the time of antibody addition but before the addition of GTP[S] were analyzed for the kinetics of ligand dissociation and are presented in Figure 4.

Equilibrium Cytometric Binding Assays. For all the fluorescent peptides employed, the cytometric binding assays used a Facscan flow cytometer (Fay et al., 1991). Briefly, a suspension of permeabilized neutrophils (10^6/mL) in freshly filtered IBB (pH 7.3 or 9.0) was equilibrated in the presence of calibrated solutions of fluorescent peptide (nominal concentrations ranging from 0.01 to 30 nM) for at least 1 h at RT. Control ("blocked") samples were made by suspending cells in IBB to which had been added excess nonfluorescent blocking peptide, CHO-Nle-Leu-Phe-Nle-Tyr-Lys (10^{-6} M). Histograms of the individual suspensions were acquired at RT in the cytometer, and data were expressed as the mean fluorescence channel of the cell suspension. Specific binding was calculated from mean fluorescence channel values of the

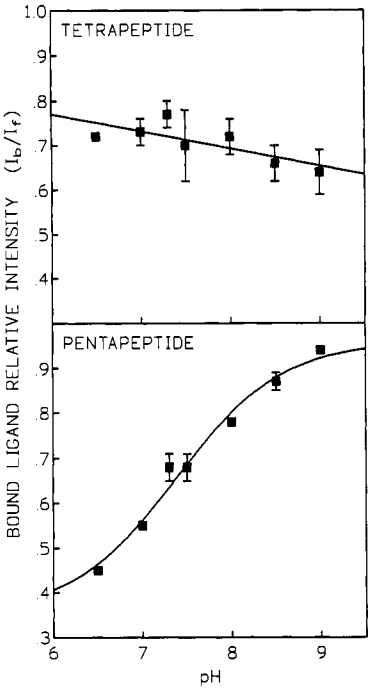

FIGURE 2: Bound fluorescent ligand intensity as a function of pH. Spectrofluorometric binding experiments with either tetrapeptide or pentapeptide were performed over a pH range from pH 6.5 to 9.0. Bound ligand fluorescence was calculated as demonstrated in eq 1. Plots represent mean bound intensity values from duplicate experiments, each performed with duplicate determinations.

bound sample minus the mean of the blocked samples, and quantitation was a described previously (Fay et al., 1991).

Kinetic Cytometric Analysis. Kinetic measurements of the dissociation of bound ligand from its receptor were performed by diluting 100-μL aliquots of cells bound to equilibrium with 0.5 nM peptide into 900 μL of IBB containing blocking peptide, quickly vortexing, and then immediately placing the sample on the cytometer. In order to acquire binding data kinetically, repetitive histograms were acquired sequentially at intervals of 15–60 s which were varied according to the steepness of the binding curve. Individual concentrations were repeated in duplicate for both bound and blocked cells.

Binding Analysis. Binding data were analyzed by nonlinear regression. Equilibrium data, bound peptides as a function of free peptide, was analyzed for the major class of high-affinity sites. In the kinetic experiments, peptide is dissociated either in the presence of excess antibody to fluorescein (spectrofluorometry) or in the presence of excess unlabeled peptide (cytometry), which precludes the rebinding of labeled peptide to cells. Thus, data were fit to simple exponential decay (Fay et al., 1991). Data representing the mole fraction of ligand bound or ligand dissociation rate constant, both as functions of pH, were fit by equations for sigmoidal curves. In all cases, data analysis used the GraphPad InPlot computer program.

RESULTS AND DISCUSSION

Fluorescence Quenching upon Binding to the Receptor as a Function of pH. The fluoresceinated ligands used in these studies exhibit a pH dependence of fluorescence intensity similar to unconjugated fluorescein and identical to conjugated FITC (Mercola et al., 1972). The ligands also had similar emission spectra and fluorescence lifetime at pH 7.3 (Fay et al., 1991). In contrast, quenching of the peptides by antibody to fluorescein via interaction with hydrophobic aromatic residues such as tryptophan (Watt & Voss, 1977) was pH

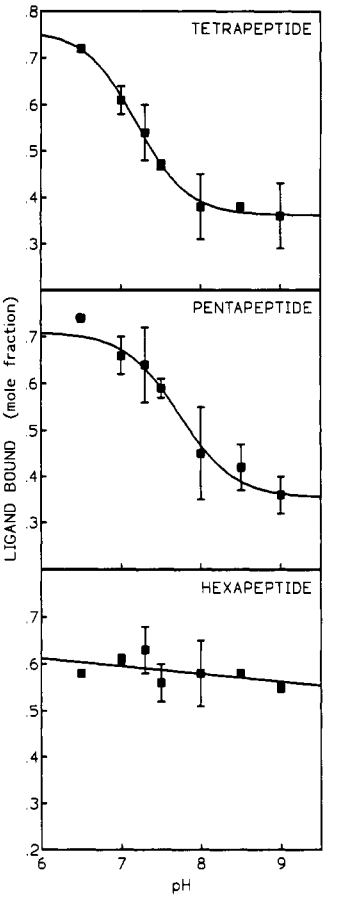

FIGURE 3: Fluorescent peptide binding as a function of pH. Spectrofluorometric binding experiments were performed as described over a pH range from pH 6.5 to 9.0 and analyzed for the mole fraction found (eq 2, Materials and Methods). Data are expressed as mean values derived from duplicate experiments, each performed with duplicate determinations.

independent with a characteristic small hydrophobic red-shifted emission (Levison et al., 1975; Herron et al., 1989).

As noted previously (Sklar et al., 1990), the fluorescent tetrapeptide and pentapeptide are quenched upon binding, while the hexapeptide is unchanged in fluorescence intensity. At pH 7.3, the pentapeptide is quenched approximately 60% more than the tetrapeptide, 45% versus 28%, respectively (Fay et al., 1991). To determine the basis for quenching upon binding to the formyl peptide receptor, binding experiments were performed over a pH range from pH 6.5 to 9.0. Permeabilized cells were used to avoid the release of oxidants and receptor internalization. Ligand binding in such preparations has previously been shown to be relatively homogeneous (Fay et al., 1991). Plots from binding experiments at extreme pH values are shown in Figure 1 and are representative of binding experiments to generate Figures 2–4. Quenching was calculated (eq 1, Materials and Methods) and plotted as a function of pH (Figure 2). While the quenching of the bound pentapeptide decreased with pH, the bound tetrapeptide was essentially pH independent. In similar experiments using the hexapeptide, no quenching upon binding was detected at any pH tested (not shown). No spectral shifts were observed upon ligand binding with either the tetrapeptide or pentapeptide in pilot spectrofluorometric experiments or in studies employing Fourier transform flow cytometry (not shown).

Ligand Binding as a Function of pH. The extent of ligand binding is shown in Figure 3 (eq 2). Both the tetrapeptide and pentapeptide demonstrated a pronounced decrease in binding as a function of increasing pH while the hexapeptide

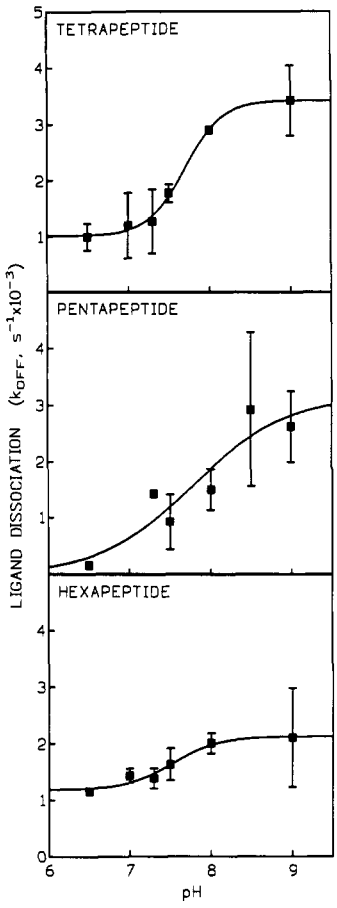

FIGURE 4: Ligand dissociation as a function of pH. Data from which representative experiments are shown in Figure 1 were analyzed for the kinetics of ligand dissociation in the absence of GTP[S]. Dissociation rate constants shown are mean values from duplicate experiments, each performed with duplicate determinations, and are plotted as a function of pH.

Table I: pH-Dependent Ligand Binding Summary[a]

	ligand dissociation		ligand binding affinity	
peptide	k_{off} (s^{-1} × 10^{-3}), pH 7.3	ratio, pH 9.0/7.3	k_D (nM), pH 7.3	ratio, pH 9.0/7.3
tetra	1.27	2.69	0.04	1.75
penta	0.93	2.82	0.03	2.67
hexa	1.38	1.52[b]	0.13	1.31

[a] Ligand dissociation rate constant data were obtained from experiments shown in Figure 4. Due to pH-dependent quenching of fluorescein, binding data could not be obtained when flow cytometric experiments were performed at low pH. In three initial experiments, binding of each of the ligands was determined at pH 7.3. In a subsequent series of experiments, the relative values at pH 7.3 and pH 9.0 were determined. The relative values were then normalized to the calibrated binding constants obtained in the earlier three experiments at pH 7.3 for each corresponding ligand. Mean values at pH 7.3 are calculated from the initial three experiments for each ligand; the ratios at pH 7.3 to pH 9.0 are calculated from the subsequent duplicate experiments. [b] Antibody to fluorescein recognizes bound hexapeptide (Sklar et al., 1990). At the antibody concentration of 25 mM used, the recognition slightly increases the absolute k_{off} values calculated.

displayed a much less pronounced decrease in binding over the same pH range. Equilibrium binding experiments with the three fluorescent ligands were also performed (data not shown), yielding plots of bound ligand as a function of free ligand concentration identical to those shown earlier (Fay et al., 1991). K_d values determined from experiments performed at pH 7.3 and 9.0 are shown in Table I. In all cases, binding affinity was lower at pH 9.0 than at pH 7.3. However, as in the spectrofluorometric binding experiments, the change in binding of the hexapeptide was less pronounced than for the other ligands.

Ligand Dissociation as a Function of pH. From data such as that in Figure 1, the rates of dissociation of ligand from cells were calculated between pH 9.0 and 6.5 (Figure 4). For all the ligands, the dissociation rate constants increased with increasing pH. Both the tetrapeptide and pentapeptide demonstrated pronounced effects of pH, k_{off} values increasing by 169% and 182%, respectively, between pH 7.3 and 9.0 (Table I). The hexapeptide displayed a much smaller effect (52%, Table I). These results were in good agreement with dissociation rate constants obtained by kinetic cytometric analysis (not shown). The changes in k_{off} for the tetrapeptide and pentapeptide were 153% and 204%, respectively. At pH 7.3 and 9.0, respectively the k_{off} values were 4.11×10^{-4} s^{-1} and 1.04×10^{-3} s^{-1} for the tetrapeptide and 1.14×10^{-3} s^{-1} and 3.47×10^{-3} s^{-1} for the pentapeptide (data not shown).

Fluoresceinated Ligands in Receptor Binding Pockets. Although the amino acid sequence of the formyl peptide receptor is known, and predictions have been made concerning the regions of the amino acid sequence in which the putative transmembrane domains of the receptor may lie (Boulay et al., 1990), little is known concerning the manner in which it interacts with ligand. The fluorescent characteristics of FITC were therefore employed to investigate the interactions of ligand within the binding pocket of the formyl peptide receptor, specifically to test for protonation of the ligand.

All three fluoresceinated peptides had essentially identical pH characteristics as derivatized FITC in solution (Mercola et al., 1972). These properties arise from protonation of the xanthonyl hydroxyl group of fluorescein (pK ~6.7). The solution fluorescence is directly related to the proportion of the dianionic species. As the pH of the experimental protocols was not below pH 6.5, only the xanthonyl hydroxyl group of fluorescein could affect fluorescence emission, since with a pK value of 4.4, the phenyl carboxyl group of the fluorescein molecules was at all times primarily in the unprotonated form. Thus, changes in fluorescence upon binding to receptor were likely to reflect fluoresceinated ligand in which the xanthonyl hydroxyl was initially in the unprotonated state.

Significance of Differences in Bound Ligand Intensity. As previously shown (Fay et al., 1991), the fluorescent tetrapeptides and pentapeptides are quenched to different extents when bound to the formyl peptide receptor. Data presented here demonstrate that while the pentapeptide had a strong positive dependence of bound intensity on pH, the tetrapeptide was relatively independent of pH. This difference defines distinct microenvironments within the binding pocket, supporting earlier work by Freer et al. (1982). Their experiments utilized formyl peptides three or four amino acids in length and of different composition.[2] Their results showed that optimal binding and biological activity were achieved when the formyl peptide was constructed of four hydrophobic amino acids, with the carboxy terminus having the ability to form hydrogen bonds. These data suggest that the receptor contains a binding pocket with a hydrophobic microenvironment into which the formylated amino terminus of the formyl peptide ligand may fit. With the fluoresceinated tetrapeptide and pentapeptide binding in this manner, the fluorescein of the shorter tetrapeptide could be positioned largely within this hydrophobic microenvironment within the receptor. Quenching of the tetrapeptide could be possible through stacking interactions

[2] The data from Freer et al. show consistency between ligand affinity and efficacy for the whole formyl peptide family. We have verified similar dose–response behavior for a fluoresceinated tetrapeptide and hexapeptide at neutral pH.

with neighboring aromatic residues, a mechanism previously shown to quench fluorescein (Watt & Voss, 1977). The fluorescein on the longer peptapeptide appears to be positioned beyond the hydrophobic region of the binding pocket, into another microenvironment which quenches by protonation. Since the reactions that are followed are those in which the ligand is in the dianionic (nonquenched) state prior to binding, all pH-dependent changes must occur within the binding pocket. Thus, the dependence of quenching as a function of pH reflects the probability, within the binding pocket, of proton donation by the receptor and proton acceptance by the ligand. To donate a proton efficiently to the xanthonyl hydroxyl group of the fluorescein, the pK of the donor would have to be greater than 6.0–7.0. From the sigmoidal behavior of the quenching of the pentapeptide in Figure 3, it appears that the binding pocket pK_a is ~7.5 (half-maximal quenching). The slightly negative pH dependence of quenching of the tetrapeptide may reflect protection of the ligand from the aqueous phase in the hydrophobic microenvironment.

Ligand Binding and Dissociation. All of the fluoresceinated formyl peptide ligands have a hydrophobic amino terminus of at least four amino acids in length. If hydrophobic interactions of the amino terminus of any of these ligands with the binding pocket were the only force involved in binding, pH would not be expected to have a large influence nor a differential effect on the ligands. As seen in Figure 3 and equilibrium binding results (Table I), the binding of all three fluoresceinated ligands is weaker with increasing pH, the effects being smallest for the hexapeptide. Because it appears that there is no additional protonation of tetrapeptide on binding to the receptor, the stabilization of binding may reflect a change in structure of the binding pocket itself. Since the tetrapeptides and pentapeptides are bound entirely within the receptor pocket, they would be more profoundly affected than the hexapeptide. Once protonated, the xanthonyl hydroxyl group of the pentapeptide could hydrogen bond with other residues in the binding pocket, even the deprotonated donor, further securing the bound ligand. This could account for the very slow dissociation rate constant specific for pentapeptide at pH 6.5 seen in Figure 4.

Implications for a Model for Ligand Binding. Taken together, data presented previously (Freer et al., 1982; Sklar et al., 1990), as well as the data presented here, suggest a model for ligand binding in which the formyl peptide receptor binding pocket (1) can accommodate at least five, but not more than six, amino acids, (2) contains at least two microenvironments, one hydrophobic and another capable of supporting protonation, both of which can affect ligand binding, and (3) can, if the receptor is protonated, undergo a conformational change, stabilizing binding. The amino acid sequence of the formyl peptide receptor and the predicted structure (Boulay et al., 1990) have features consistent with this model. The predicted transmembrane regions of the receptor are especially rich in hydrophobic residues, potentially forming the hydrophobic microenvironment of the binding pocket 2–3 α-helical turns down from the extracellular loops. Extracellular domains 2 and 3 also have clusters of hydrophobic residues which contain aromatic tryptophan and phenylalanine residues. These clusters not only could form a hydrophobic microenvironment but also could quench fluorescein independently of pH, as is seen in the tetrapeptide. In extracellular domain 2, at amino acid position 90, there is a histidine residue that could quench the pentapeptide by protonation with half-maximal quenching at approximately pH 7.5 (Figure 2). Since the midpoint for binding and dissociation behavior (Figures 3 and 4) is near pH 7.5, it is possible that this histidine may be responsible for these pH-dependent changes as well. A histidine has been previously suggested to influence ligand binding (Spilberg et al., 1986) with a pK_a between 7.0 and 7.5. Extracellular domains 3 and 4 have a cluster of lysine, arginine, and glutamic and aspartic acids in close proximity, which also should not be excluded in receptor protonation.

These present approaches begin to define the formyl peptide receptor binding pocket at natural abundance. Refinements to the approach should include (1) the use of peptide ligands conjugated with fluorescent dyes having a more distinct spectral response to environmental conditions, rather than the general change in emission intensity seen in fluorescein, (2) the development and utilization of more sophisticated instrumentation capable of detecting spectral changes in these new probes when used in cells with receptors at natural abundance, (3) the ability to resolve structural changes in the receptor associated with signal transduction, and (4) the use of molecular biology to replace amino aspects suspected of contributing to spectral responses to begin to align three-dimensional elements of the binding pocket.

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