On the pH Dependence of Amide Proton Exchange Rates in Proteins

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ABSTRACT We have analyzed the pH dependencies of published amide proton exchange rates (k_{ex}) in three proteins: bovine pancreatic trypsin inhibitor (BPTI), bull seminal plasma proteinase inhibitor IIA (BUSI IIA), and calbindin D_{9K}. The base-catalyzed exchange rate constants (k_{OH}) of solvent exposed amides in BPTI are lower for residues with low peptide carbonyl exposure, showing that the environment around the carbonyl oxygen influences k_{OH}. We also examined the possible importance of an exchange mechanism that involves formations of imidic acid intermediates along chains of hydrogen-bonded peptides in the three proteins. By invoking this "relayed imidic acid exchange mechanism," which should be essentially acid-catalyzed, we can explain the surprisingly high pH_{min} (the pH value at which k_{ex} reaches a minimum) found for the non-hydrogen-bonded amide protons in the β-sheet in BPTI. The successive increase of pH_{min} along a chain of hydrogen-bonded peptides from the free amide to the free carbonyl, observed in BPTI, can be explained as an increasing contribution of the proposed mechanism in this direction of the chain. For BUSI IIA (pH 4–5) and calbindin D_{9K} (pH 6–7) the majority of amide protons with negative pH dependence of k_{ex} are located in chains of hydrogen-bonded peptides; this situation is shown to be consistent with the proposed mechanism.

INTRODUCTION

As was suggested by Linderstrøm-Lang in 1955, amide proton exchange has turned out to be a very useful tool for the study of protein structure in solution. To fully interpret exchange data, a thorough understanding of the underlying physical mechanisms is needed, and we have studied the consequences of some of the common models for the pH dependence of exchange rates of individual amide protons in proteins. This dependence has been measured for a variety of proteins, e.g., in BPTI (Wüthrich and Wagner, 1979; Richarz et al., 1979; Wagner and Wüthrich, 1979; Hilton and Woodward, 1979; Wüthrich et al., 1980; Woodward and Hilton, 1980; Hilton et al., 1981; Tüchsen and Woodward, 1985a,b; Tüchsen and Woodward, 1987; Tüchsen et al., 1987; Gallagher et al., 1992), calbindin D_{9K} (Skelton et al., 1992b), lysozyme (Delepierre et al., 1987; Pedersen et al., 1991, 1993; Thomsen and Poulsen, 1993) and in BUSI IIA (Wüthrich et al., 1984). For model compounds, e.g., poly-D,L-alanine (Hvidt, 1973), the pH dependence of the amide proton exchange rate (k_{ex}) can be expressed according to Woodward and Hilton (1980):

$$k_{ex} = k_H[H^+] + k_{OH}[OH^-] + k_0 \qquad (1)$$

where k_H, k_{OH}, and k_0 are the rate constants for acid-catalyzed, base-catalyzed, and direct exchange with water, respectively. Direct exchange with water (k_0) is negligible for model compounds. Amide proton exchange rates reach a minimum (k_{min}) at pH ~3 (pH_{min}) for model compounds (Molday and Kallen, 1972; Molday et al., 1972; Hvidt, 1973). On either side of pH_{min}, $\Delta lg(k_{ex})/\Delta pH$ is close to -1 for $pH < pH_{min}$ and to 1 for $pH > pH_{min}$ for model compounds, indicating a close to first order dependence of the exchange rate on pH in these pH ranges.

pH_{min} for an amide group reflects the ratio between its acid- and base-catalyzed exchange rate constants, according to Leichtling and Klotz (1966):

$$pH_{min} = \frac{1}{2}[pK_w + 1g(k_H/k_{OH})] \qquad (2)$$

where K_w is the ionization constant of water. Thus, shifts of pH_{min} in relation to model compounds is a consequence of unequal effects on the acid- and base-catalyzed exchange rate constants. That is, a peptide in an environment giving rise to a higher k_H and/or a lower k_{OH} than those of model compounds obtains a pH_{min} >3 and vice versa.

However, $\Delta lg(k_{ex})/\Delta pH$ for solvent-protected amide protons in proteins is often smaller than unity for the base-catalyzed exchange rate, the pH minimum of the exchange rate is often more shallow and broad, and pH_{min} deviates from that of model compounds. Many different physical models have been suggested for the exchange reaction scheme that can account for the deviations of the pH dependence of the exchange rates from that of model compounds. According to the Linderstrøm-Lang model (Linderstrøm-Lang, 1955; Hvidt and Nielsen, 1966) the exchange rate of a solvent protected amide is determined by the opening and closing rates of exposure of the amide proton to the solvent, where it is exchanging according to Eq. 1. According to the "breathing" model (Wagner and Wüthrich, 1982; Wüthrich et al., 1984; Englander et al., 1983, 1992; Kuwajima and Baldwin, 1983; Wand et al., 1986), the

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Abbreviations used: BPTI, bovine pancreatic trypsin inhibitor; BUSI IIA, bull seminal plasma protease inhibitor IIA; O, carbonyl oxygen; NH, amide proton.

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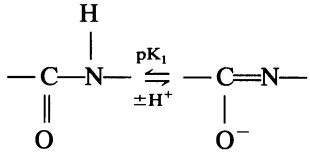
amide groups become accessible to the solvent by local unfolding of the protein. According to the solvent penetration model (Woodward and Rosenberg, 1971; Ellis et al., 1975; Lumry and Rosenberg, 1975; Woodward, 1977; Richards, 1979), water molecules and ions penetrate buried regions of the protein via small amplitude motions of the protein atoms, which may form channels into the interior of the protein. According to the multistate model (Wagner and Wüthrich, 1979), the exchange is taking place because of global fluctuations of the protein. Another explanation that has been given for the non-first order pH dependence of the base-catalyzed exchange and the broad, shallow minima observed for the exchange rate, especially for deeply buried protons, is that catalysis by water molecules (k_0 in Eq. 1) contributes to the exchange rate (Gregory et al., 1983).

The acid- or base-catalyzed exchange of an amide proton can in principle occur via three mechanisms (Perrin, 1989), which we describe briefly below, before further discussing the pH dependence of the exchange rate and the various models.

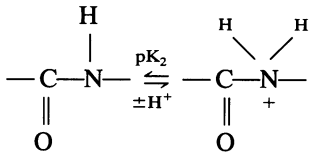
Base-catalyzed exchange, where a hydroxide ion removes the amide proton to produce the imidate anion, which is subsequently reprotonated:



(3)

The pK_a for deprotonation (pK_1) of the amide group (Molday and Kallen, 1972) in *N*-metylacetamide has been estimated to 18.

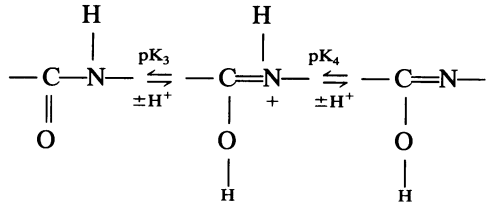
Acid-catalyzed N-protonation:



(4)

with a pK_a value for protonation of the amide group (Fersht, 1971; Molday and Kallen, 1972) (pK_2) around −7.

The "imidic acid mechanism," where a protonation of the carbonyl oxygen acidifies the amide proton so that a water can remove it to produce the imidic acid, which returns to the amide through the reverse reaction:



(5)

The experimental pK_a for protonation of the carbonyl oxygen (Fersht and Jencks, 1970) (pK_3) is ~0, and the pK_a for N-deprotonation of the O-protonated imidic acid (pK_4) has been estimated to 7.5 for *O,N*-dimetylacetimidate (Pletcher et al., 1968). Perrin and Arrhenius (1982) have shown that the imidic acid mechanism is preferred over the N-protonation mechanism for acid-catalyzed exchange of amides protons in proteins. In the transition state of the imidic acid mechanism (Eq. 6), the positive charge will be strongly shifted toward the nitrogen because of the different basicity of O and N (Molday and Kallen, 1972).

$$H—(O{=}C{=}N)^+—H \qquad (6)$$

The transition state of the N-protonation mechanism (Eq. 4) bears a more localized charge on the nitrogen. It will be more destabilized by electron-withdrawing acyl substituents, including peptides, in favor of the imidic acid mechanism (Perrin, 1989). Moreover, Tüchsen and Woodward (1985b) found a striking correlation between the acid-catalyzed exchange rate constant (k_H in Eq. 1) and the solvent exposure of the carbonyl oxygen of the same peptide (i.e., of the previous residue) of amide proteins at the solvent-protein interface. This correlation strongly suggests that the imidic acid mechanism is dominating the acid-catalyzed exchange. Also, agreement between electrostatic calculations and the ionic strength dependence of the exchange rate, assuming Eq. 5 to be dominating for the acid-catalyzed exchange, supported the dominance of the imidic acid mechanism (Tüchsen and Woodward, 1985b).

Because the imidic acid mechanism reverses the hydrogen bond donor/acceptor properties of the backbone, both the carbonyl oxygen and the amide proton must be at least temporarily accessible to the solvent in order to interact with the water molecules. This could be achieved by the natural "breathing" of the protein, such as local unfolding of α-helical fragments or by a temporary breaking of the hydrogen bonds of β-sheet structures (Perrin, 1989).

In this work, we will analyze the pH dependence of published exchange rates of amide protons in BPTI by Richarz et al. (1979), Tüchsen and Woodward (1985a and 1987) and Tüchsen et al. (1987) where k_{min}, k_H, k_{OH}, and pH_{min} have been estimated for most of the residues. We will assume that the imidic acid mechanism is dominating the acid-catalyzed exchange. For the most rapidly exchanging amide protons in BPTI, we observe also that k_{OH} decreases with decreased solvent accessibility of the peptide carbonyl oxygen.

Tüchsen and Woodward (1985b) proposed a mechanism where the exchange of more buried protons occurs by charge delocalization through chains of hydrogen-bonded peptides (Fig. 1). The motivation for suggesting this mechanism, which we will call "the relayed imidic acid exchange mechanism," was that only water molecules would have to penetrate into more buried regions for exchange to occur. In the exchange mechanisms described in Eqs. 3–5, charged species (OH^- and H^+) are required to contact the peptide. According to the solvent penetration model (Woodward and Rosenberg, 1971; Ellis et al., 1975; Lumry and Rosenberg, 1975; Woodward, 1977; Richards, 1979), these ions must either penetrate in a hydrated state (being very large), or stripped of their hydrating waters, which would be an en-

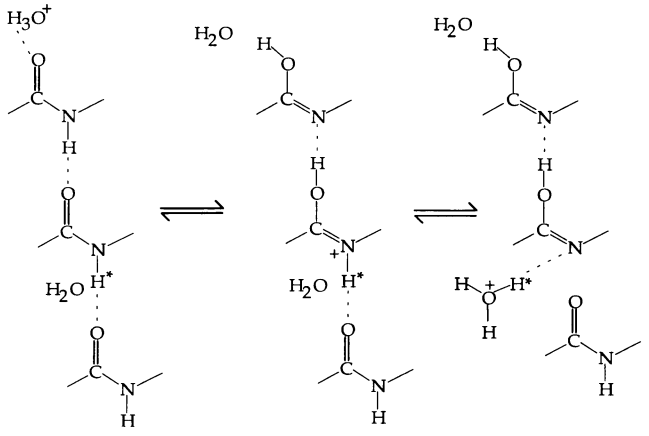


FIGURE 1 The relayed imidic acid exchange mechanism for exchange of the amide protons participating in chains of peptide hydrogen bonds, proposed by Tüchsen and Woodward (1985b).

ergetically costly process. The mechanism (Fig. 1) also requires a charge separation, but H_3O^+ does not have to penetrate into the protein interior for the more buried amide protons. The relayed imidic acid exchange mechanism has also been proposed for translocation of protons in transmembrane proteins (Kayalar, 1979). The loss of resonance energy (Fersht, 1971) upon formation of the intermediate enol forms (Fig. 1) is an argument against this mechanism. On the other hand, the π-bond cooperativity along chains of N — H · · · O = C hydrogen bonds shifts the keto-enol tautomerization toward the enol form, which is seen as a shortening of the C-N bond and a lengthening of the C-O bond compared with a free peptide (Jeffrey and Saenger, 1991). Nuclear one-bond $^1J_{NC}$ coupling constants measured in nuclear magnetic resonance (NMR) experiments on ^{13}C- and ^{15}N-labeled model compounds and on the protein ubiquitin also reveal a cooperative polarization of peptide bonds along chains of N — H · · · O = C hydrogen bonds (Juranic et al., 1995).

Here we investigate the possible importance of the relayed imidic acid exchange mechanism in BPTI by examining the variation of pH_{min} along chains of hydrogen-bonded peptides. From the pH dependence of amide proton exchange in the proteinase inhibitor IIA from bull seminal plasma (BUSI IIA) between pH 4.0 and 4.9 (Wüthrich et al., 1984) and in calbindin D_{9K} between pH 6.0 (Skelton et al., 1992b) and 7.25 (Linse et al., 1990), we examine the variation of $\Delta lg(k_{ex})/\Delta pH$ along chains of hydrogen-bonded peptides. If k_{OH} is sufficiently low and/or k_H is sufficiently high, pH_{min} will be shifted above the two respective pH intervals above and result in a negative pH dependence (see Eq. 2). An estimate of $\Delta lg(k_{ex})/\Delta pH$ for an amide proton in a certain pH interval thus gives information whether pH_{min} is above or below this pH range. We find that many of the residues participating in chains of hydrogen-bonded peptides (C = O · · · H — N) have a negative pH dependence of the proton exchange rate in both BUSI IIA and in calbindin D_{9K}. This can possibly be explained by the action of the relayed imidic acid exchange mechanism, which might play a role for exchange of amide protons that participate in a chain of peptide hydrogen bonds in α-helices and, in particular, in β-sheets. We study the amide/carbonyl groups for the three proteins above taking into consideration 1) hydrogen bonding, 2) solvent exposure, 3) distance from the protein-solvent interface, and 4) possible ion coordination, and try to correlate these properties with the pH dependence or the pH_{min} of the exchange rate.

METHODS

BPTI

Because the measurements of the amide proton exchange rates were performed in solution, the calculations of structural data from the NMR structures seem more appropriate than using the x-ray structures of the proteins. An average structure was calculated from the 20 refined NMR solution structures of BPTI at pH 4.6 (Berndt et al., 1992) and energy-minimized within the program package CHARMM (Brooks et al., 1983) to remove unfavorable contacts that develop when the structures are averaged. Hydrogen bonds were taken from the analysis by Berndt et al. (1992). The pH_{min} (i.e., the pH values where the exchange rate reaches a minimum) of the amide protons in BPTI were taken from Richarz et al. (1979), Tüchsen and Woodward (1985a and 1987) and Tüchsen et al. (1987).

BUSI IIA

Structural characteristics of BUSI IIA were calculated from the NMR solution structure (Williamson et al., 1985) at pH 4.3 and 45°C. The hydrogen bonds in BUSI IIA were assigned using the criteria that the acceptor (A)-donor proton (H-D) distance is <2.5 Å, and the angle A–H-D is >135°. The exchange rates of the amide protons in BUSI IIA at pH 4.0 and 4.9 have been measured by two-dimensional correlated ^1H NMR spectroscopy (Wüthrich et al., 1984).

Calbindin D_{9K}

For calbindin D_{9K} we used the minimized average NMR solution structure (Kördel et al., 1993) to calculate structural characteristics. The wild type calbindin D_{9K} consists of a mixture of the *cis*- and *trans*-isomers about the Gly42-Pro43 bond (Chazin et al., 1989), and Pro43 was mutated to a glycine before the structure determination (Kördel et al., 1993) to facilitate interpretation of experimental data. To obtain reasonable positions for the two calcium ions in the calcium-binding loops (Szebenyi and Moffat, 1986), we superimposed the *trans*-Pro43 isomer of the 1.6 Å resolution crystal structure (Svensson et al., 1992) of the calcium-loaded calbindin D_{9K} on the NMR structure. The superposition was carried out by minimizing the root mean square deviation of the non-hydrogen atoms between the two structures and the calcium ions from the crystal structure were subsequently placed in the solution structure. This procedure is justified, because the two structures are very similar to each other (Kördel et al., 1993). The hydrogen bonds were taken from the analysis by Kördel et al. (1993). Amide proton exchange rate measurements in calbindin D_{9K} at pH 7.25 (Linse et. al., 1990) were performed as a saturation transfer experiment on the wild type, i.e., on the mixture (Chazin et al., 1989) of the *cis*- and *trans*-isomers of Pro43. The exchange rate measurements at pH 6.0 (Skelton et al., 1992b) were carried out on the Pro43Gly mutant using ^{15}N-enriched protein, in which the exchange was followed after dissolution in D_2O.

Solvent exposure

We estimated the solvent accessible surface area (a.s.a.) for peptide amides and carbonyls in the three proteins using a probe with a radius of 1.6 Å

around, according to the algorithm by Lee and Richards (1971), which is available within the CHARMM program package (Brooks et al., 1983). Fractional, percentile a.s.a. values were obtained by dividing the a.s.a. by the accessible surface area of an amide group/carbonyl oxygen of a free peptide.

As a measure of the proximity of a protein atom to the protein-solvent interface, we calculated the distance from a protein atom to the nearest hypothetical water molecule (d_{HWM}, Pedersen et al., 1991). d_{HWM} serves as a crude measure of the solvent accessibility for peptides, which have 0 a.s.a. of the amide/carbonyl group. The positions of the HWMs were determined by dividing the space covered by and surrounding the protein into a lattice with grids of 1 Å spacing. If a sphere of radius 1.6 Å, representing the water molecule, could be placed without overlapping the protein with its van der Waals radius, an HWM was positioned at this point. Thereafter, the shortest distances from the amide nitrogens and the carbonyl oxygens to the HWMs were calculated for each residue (d_{HWM}).

RESULTS AND DISCUSSION

Protonation of the carbonyl (pKa ~ 0) is more probable than a deprotonation of the amide nitrogen ($pK_a \sim 18$) for pH values less than ~9, which is considerably higher than pH_{min} for k_{ex}. Since a protonation of the carbonyl oxygen lowers pK_a for deprotonation of the amide group from 18 to ~7.5 (Pletcher et al., 1968; Molday and Kallen, 1972), and since the base-catalyzed exchange mechanism (Eq. 3) is dominating for $pH > pH_{min}$, we expect that k_{OH} is also affected by the solvent exposure of the carbonyl. The dependence of k_H on the carbonyl solvent exposure was shown by Tüchsen and Woodward (1985b), and we will show that a similar variation also applies for k_{OH}, as will be illustrated for BPTI below.

In the relayed imidic acid exchange mechanism, the proton exchange proceeds as in the imidic acid mechanism, i.e., with an initial protonation of the free carbonyl at one end of a chain of hydrogen-bonded peptides (Fig. 1). The corresponding peptide amide proton, which now is acidified, may in turn protonate another carbonyl oxygen to which it is hydrogen bonded. In this way proton exchange is relayed through the chain of hydrogen-bonded peptides, and an acidic amide proton somewhere along the chain can exchange with water molecules that have penetrated into more buried regions of the protein.

The mechanism implies an electron transport through the chain, which can be expected to be facilitated by constructively interacting dipole moments of the peptide bonds in the chain and by π-bond cooperativity (Jeffrey and Saenger, 1991). These effects increase the pK_a for protonation of the free carbonyl and reduce the pK_a for deprotonation of the free amide group at the other end of the chain. The reversal of the mechanism, i.e., a deprotonation of the free amide group with a subsequent charge delocalization along the chain of peptides, would in principle also be feasible. However, the increased pK_a for protonation of the free carbonyl oxygen and the corresponding reduction of the pK_a for deprotonation of the free amide group in a chain of peptide hydrogen bonds, should retain the relative probabilities of these two events. Considering that protonation of O is more probable than deprotonation of the amide group for pH values less than ~9, the former event would be more probable up to this pH value. We therefore expect the action of the relayed imidic acid exchange mechanism to result in an increase of pH_{min}. In chains with electrostatic surroundings that further acidify the free amide group in the chain and also block protonation of the free carbonyl oxygen, e.g., the presence of Ca^{2+} in calbindin D_{9K} (see below), a reversal of the mechanism might be feasible.

The relative importance of the relayed imidic acid exchange mechanism should be increased by the following factors: 1) strong peptide hydrogen bonds that facilitate the formation of the imidic acid intermediates, 2) low solvent exposure of hydrogen-bonded carbonyl groups along a chain in the interior of the protein, because an exposed carbonyl oxygen would accept a proton from the solution rather than from the hydrogen-bonded amide group, 3) a short distance from the amide proton to exposed carbonyl oxygens along the chain because the probability of forming chains of imidic acids (Fig. 1) increases when few peptide hydrogen bonds are involved in the intermediate state, and 4) high solvent exposure of the free carbonyl group at one end of the chain that facilitates protonation of the oxygen, which initiates the relayed imidic acid exchange mechanism.

We have identified chains of hydrogen-bonded peptides that would allow the relayed imidic acid exchange mechanism to operate in three proteins (BPTI, BUSI IIA, and calbindin D_{9K}) for which exchange rate data is available. The factors described above were inspected for each peptide amide and carbonyl in these chains in the three proteins using the accessible surface areas (% a.s.a) and distances to the closest possible location of a water molecule (d_{HWM}) as measures of solvent exposure.

BPTI

The variation of k_{OH} (corrected for electrostatic effects) of the most rapidly exchanging amide protons in BPTI with the fractional solvent exposure of the amide groups (SA_{NH}) has been investigated and compared with k_{OH} of poly-D,L-alanine (Hvidt, 1973) multiplied with SA_{NH} (see Fig. 4 in Tüchsen and Woodward, 1985b). The amide protons of groups with hydrogen-bonded carbonyls can be divided in two classes, one with larger k_{OH} values and one with lower k_{OH} values than expected from the model compound. The amide protons of the former class are essentially located at the N-termini of the helices. Their increased k_{OH} in relation to a model compound with the same amide solvent accessibility could be explained by the inductive effects on the hydrogen-bonded carbonyls from the helix dipole, increasing the acidity of the nitrogen and hence k_{OH} (Tüchsen and Woodward, 1985b). The latter group, with k_{OH} values less than expected from model compounds, consists solely of amide protons from the central β-sheet, i.e., Gly12, Ile19, Ala25, Cys30, Thr32, and Val34.

To rationalize the k_{OH} values of residues in this group, we selected residues with non-hydrogen-bonded amide protons,

with a finite solvent exposure or with a small d_{HWM} of the amide group, $d_{HWM}(N) \leq 3.0$ Å, to be confident that the amide proton is readily accessible to the solvent. For these residues, we examined the variation of $\lg(k_{min})$, $\lg(k_{OH})$, and $\lg(k_H)$ with $d_{HWM}(O)$ (see Fig. 2). k_{min} serves as a measure of the overall exchange rate, since it has equal contributions from the acid- and base-catalyzed exchange rates. There is a clear trend of decreasing $\lg(k_{min})$ with increasing $d_{HWM}(O)$ for the residues with exposed amide groups. Free peptides (Fig. 2, *open circles*) generally have low $d_{HWM}(O)$ and exchange rapidly, whereas amide protons from predominantly the β-sheet (Gly12, Ile19 and Ala25) and one amide proton from the C-terminal helix (Ala48) have high $d_{HWM}(O)$ with slower exchange rates (Fig. 2, *top*). The residue that deviates most from this trend is Arg39 (Fig. 2), which is also different in that Cys38-O forms a hydrogen bond to the side chain of Tyr-35 (Berndt et al., 1992).

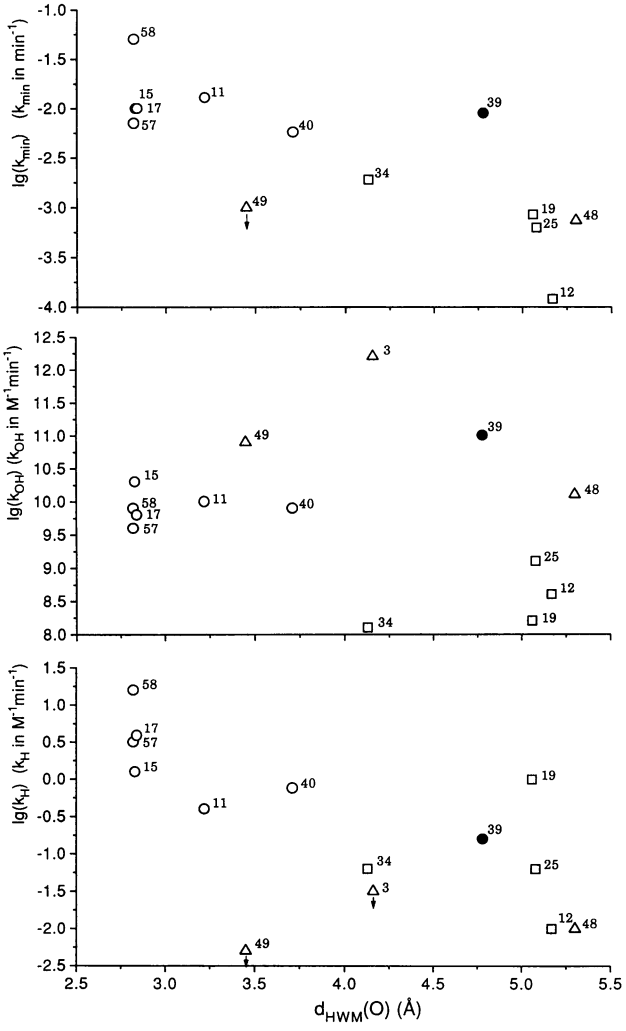


FIGURE 2 Variation of $\lg(k_{min})$ (*top*), $\lg(k_{OH})$ (*middle*) and $\lg(k_H)$ (*bottom*) (Tüchsen and Woodward, 1985a) with d_{HWM} (i.e., the distance from the nearest HWM; see Methods section) of the peptide carbonyl oxygens for residues with a finite amide group solvent exposure or with $d_{HWM}(N) \leq 3.0$. Symbols: (○) free peptides; (●) carbonyl oxygen hydrogen bonded to a side chain; (△) α-helical and (□) β-sheet peptides. Upper limits are indicated by arrows.

The variation of $\lg(k_{OH})$ shows a similar trend, but the different types of residues are more grouped. Free peptides have $\lg(k_{OH})$ values close to that of poly-D,L-alanine (9.8; Hvidt, 1973), whereas residues belonging to the β-sheet, which have deeply buried carbonyls, show a considerable (10–100-fold) decrease in their k_{OH} values. One explanation for the observed decrease is that the partial negative charge on the oxygen in the imidate ion intermediate (Eq. 3) must be solvated. This is unfavorable if that oxygen is buried in a less polar environment, which thus would lower k_{OH}. Another, quite similar explanation for this behavior of k_{OH} would be that a protonation of the carbonyl oxygen facilitates a base-catalyzed exchange of the amide proton. As was argued above, an O-protonation ($pK_a \sim 0$) would be more probable than a "direct" N-deprotonation ($pK_a \sim 18$), for pH values less than around 9, which is well above pH_{min}. The residues Asp3, Ala48, and Glu49 at the N-termini of the helices exchange with a higher k_{OH} than for model compounds (Fig. 2, *middle*) because of the aligned dipoles of a helix that further acidifies the amide nitrogen. The increased k_{OH} could possibly also be an effect of fraying ends of the helix. In a 40 ps molecular dynamics simulation of BPTI (Berendsen et. al., 1986), the N-termini of the helices were found to be more flexible than the internal parts of the helix, especially the first (N-terminal) helix. The low probability of finding a hydrogen bond between Cys5-NH and Pro2-O in the 20 refined NMR structures (Berndt et al., 1992) also indicates that the first helix is fraying. However, because the k_H values of the residues at the N-termini of the helices are very low (Fig. 2, *bottom*), the effect of fraying helices on k_{OH} is probably minor. Fraying helix ends would be seen as an increased acid-catalyzed exchange rate constant because of facilitated protonation of the carbonyl oxygen, according to the imidic acid exchange mechanism. Because of the increased acidity of the N-terminal amide protons in the helices, a solvation of the oxygen in the imidate ion intermediate or a prior protonation of the amide carbonyl oxygens would be expected to have less influence on the base-catalyzed exchange rate than in a free peptide or in a β-sheet. The electrostatic field generated by the parallel dipoles in a helix also affects the surrounding residues. This effect might be seen at the two residues closest to the C-terminal helix; Ser47 has an unusually low pH_{min} (1.7; Tüchsen and Woodward, 1985a) for a non-hydrogen-bonded peptide, and Lys46 belongs to the β-sheet but has acquired a very low pH_{min} (<1; see Fig. 3), probably because of the positive local electrostatic field and because it is terminating a chain of peptide hydrogen bonds.

Focusing on the amide protons that belong to chains of peptide hydrogen bonds (Fig. 3 and Table 1), some general features can be seen. First, the pH_{min} values increase along the chain of peptide hydrogen bonds in the direction from the free amide group to the free carbonyl group, with two minor exceptions at Tyr21-Phe33 and at Tyr23-Gln31 (see Fig. 3). The free carbonyl oxygens in the chains of peptide

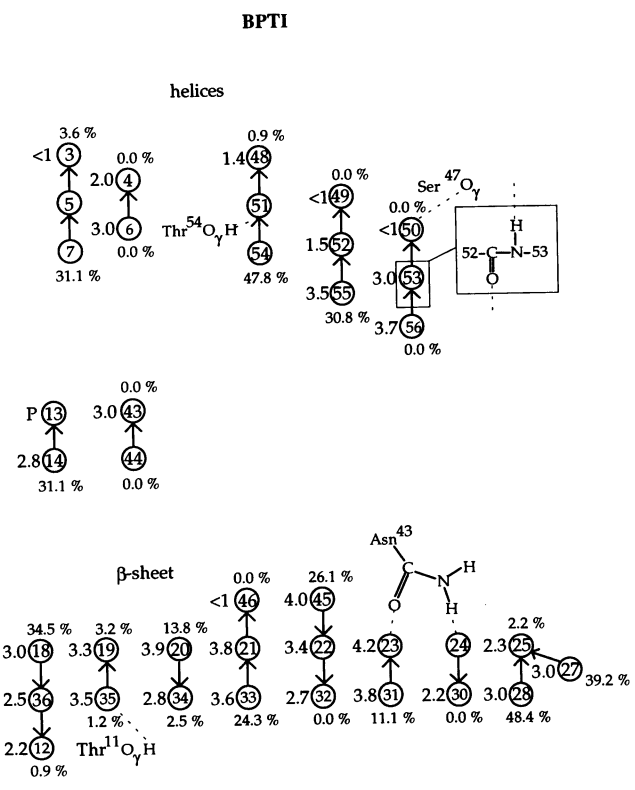


FIGURE 3 Residues participating in chains of peptide hydrogen bonds in BPTI. The pH_{min} values (Richarz et al., 1979; Tüchsen and Woodward, 1985a, 1987; Tüchsen et al., 1987) are shown beside the residue number (prolines are shown as "P"). The arrows point from the amide protons to the peptide carbonyl oxygen of the next residue in the chains, as indicated in the square. The % a.s.a. values of the free amide groups/carbonyl oxygens are shown at the ends of the chains.

hydrogen bonds (Fig. 3) are highly exposed to the solvent, except for Cys5, Asn43, Cys55, and possibly Val34 (Fig. 3, Table 1). Second, the amide protons of the free amide ends of peptide hydrogen bond chains in the central β-sheet have surprisingly high pH_{min} values considering the relative accessibilities of the peptide amide group and carbonyl oxygen. The hydrogen bonds in these chains are also strong, revealed as extremely low exchange rates (Richarz et al., 1979, Gregory et al., 1983), e.g., Tyr35-NH···Ile18-O, Arg20-NH···Phe33-O, and Phe22-NH···Gln31-O. The pH_{min} values are in the same range as for a free peptide, with both its carbonyl oxygen and amide proton exposed (~3). The amide protons of these residues are, except for Cys30, readily accessible to the solvent, which is seen as a finite solvent exposure or a low d_{HWM}(N), whereas the carbonyl oxygen is deeply buried (high d_{HWM}(O) values, see Table 1). The high pH_{min} values might be explained by a hindered solvation of the oxygen in the imidate ion intermediate (Eq. 3) or by a hindered protonation of the carbonyl group. That would decrease k_{OH} as well as k_H and therefore result in a lower overall exchange rate (see Fig. 2, *top*) with the same ratio of k_H/k_{OH} (i.e., pH_{min}) as for a free peptide. However, the residues Ile19 and Val34 both have very low k_{OH} values (Fig. 2, *middle*) but a corresponding lowering of k_H is not observed (Fig. 2, *bottom*). This is especially pronounced for Ile19, with a k_H value close to that of free peptides.

TABLE 1 Summary of structural data (Berndt et al., 1992) and pH_{min} for the amide protons in BPTI

	d_{HWM}		% a.s.a.		
Residue	N	O*	N	O*	pH_{min}
Asp3	3.6	4.2	3.6	0.0	<1‡
Phe4	3.6	3.2	0.0	3.7	2.0‡
Cys5	5.8	5.1	0.0	0.0	
Leu6	4.6	4.6	0.0	0.0	3.0§
Glu7	3.4	2.8	0.0	31.1	
Gly12	3.0	5.2	0.9	0.0	2.2‡
Cys14	3.2	2.8	0.0	31.1	2.8¶
Ile18	4.3	2.9	0.0	34.5	2.95‖
Ile19	3.0	5.1	3.2	0.0	3.3‡
Arg20	4.8	2.8	0.0	13.8	3.85‖
Tyr21	5.7	5.5	0.0	0.0	3.75‖
Phe22	7.3	6.5	0.0	0.0	3.35‖
Tyr23	6.6	6.9	0.0	0.0	4.25‖
Asn24	4.9	3.9	0.0	0.0	
Ala25	3.3	5.1	2.2	0.0	2.3‡
Ala27	3.8	2.9	0.0	39.2	3.0‡
Gly28	3.4	2.8	0.0	48.4	3.0**
Cys30	4.9	6.3	0.0	0.0	2.2‡
Gln31	4.4	3.0	0.0	11.0	3.80‖
Thr32	3.2	5.0	0.0	0.0	2.7‡
Phe33	4.0	2.8	0.0	24.3	3.60‖
Val34	3.1	4.1	2.5	0.0	2.8‡
Tyr35	5.2	3.1	0.0	1.2	3.40‖
Gly36	4.8	4.9	0.0	0.0	2.5‖
Asn43	5.1	2.9	0.0	5.2	3‡
Asn44	4.6	3.6	0.0	0.0	
Phe45	4.0	2.8	0.0	26.1	4.0‖
Lys46	4.0	4.5	0.0	0.0	<1‡
Ala48	3.3	5.3	0.9	0.0	1.4‡
Glu49	3.0	3.4	0.0	0.0	<1‡
Asp50	3.9	3.5	0.0	0.0	<1‡
Cys51	6.0	4.0	0.0	0.0	
Met52	5.2	5.5	0.0	0.0	1.5‖
Arg53	3.6	3.1	0.0	4.1	3.0**
Thr54	3.3	2.8	0.0	47.8	
Cys55	3.2	2.8	0.0	30.8	3.45‖
Gly56	3.0	3.7	3.1	0.0	3.7**

Only residues participating in chains of hydrogen-bonded peptides are tabulated, excluding the proline.

* The carbonyl oxygen of the same peptide, i.e., of the preceding residue; ‡ Tüchsen and Woodward (1985a), 25°C; § Tüchsen and Woodward (1985a), 30°C; ¶ Tüchsen et al. (1987), 25°C; ‖ Richarz et al. (1979), 45°C; ** Tüchsen and Woodward (1987), 25°C.

The relayed imidic acid exchange mechanism (Fig. 1) could possibly explain the shift of pH_{min} along the chain of peptide hydrogen bonds and the high pH_{min} values of the free amide ends in the central β-sheet chains of peptide hydrogen bonds. The free carbonyl oxygen at one end of the peptide hydrogen bond chain is highly exposed to the solvent and relatively acidic because of the aligned dipoles of the peptides in the chains, and it could readily accept a proton from the solvent; an electron would be transported through the chain to form an intermediate "imidic-acid chain" (Fig. 1). This leads to the loss of an amide proton somewhere along the chain; the proton can be exchanged

with a water molecule inside the protein. This event should be more probable closer to the free carbonyl because of the high energy tautomeric enol form that temporarily has to be formed (see Fig. 1). The successive decrease of pH_{min} from the free carbonyl to the free amide along the chain then follows, because a lower activity of the relayed imidic acid exchange mechanism would shift pH_{min} downward.

BUSI IIA

Of the 40 residues at which it was possible to obtain an estimate of $\Delta lg(k_{ex})/\Delta pH$ from k_{ex} data at pH 4.0 and 4.9 (Wüthrich et al., 1984), 15 residues had exchange rates with a weak negative (0 to −0.5) pH dependence and two (Lys14 and Gly26) had a more negative (<−0.5) pH dependence, i.e., 45% of the measured $\Delta lg(k_{ex})/\Delta pH$ values were negative. The majority of residues with negative $\Delta lg(k_{ex})/\Delta pH$ in BUSI IIA (12 of 17) are involved in formations of chains of peptide hydrogen bonds, and 6 of these 12 residues belong to peptide hydrogen bonds in the β-sheet (Fig. 4). In the α-helix, residues Ala37-Val42 have negative $\Delta lg(k_{ex})/\Delta pH$ values, whereas Cys36, Met43, and Lys44 have weakly positive pH dependencies.

Ala8 is the first residue (counting from the N-terminal) for which exchange rates at the two pH values fall below the measurable upper limit ($lg(k_{ex})$ <-2.8, k_{ex} in s^{-1}, Wüthrich et al., 1984). It is also the first residue (counting from the N-terminal) with zero solvent exposure of the carbonyl group of the same peptide; d_{HWM} is 4.9 Å, indicating that the carbonyl group is rather far away from the solvent, whereas the solvent exposure of the amide group is 1% (Table 2). Thus, as was also the case for the residues in BPTI with an exposed amide group and a buried carbonyl group, a decrease in the exchange rate (more than 10-fold for Ala8, compared with the preceding residues) can be observed if the carbonyl oxygen is deeply buried. A similar situation is found for Thr31, where the amide proton has a non-zero solvent exposure and the carbonyl oxygen of the same peptide has a d_{HWM} of 4.2 Å; the exchange rate for this residue is slightly below the upper limit of measurable exchange rates at pH 4.0 (Table 2). Gly26 and Lys52 also belong to this chain of hydrogen-bonded peptides, and both these amide protons exchange with a $\Delta lg(k_{ex})/\Delta pH$ around −0.5 (Table 2, Fig. 4). The free carbonyl oxygen (Leu51-O) has a solvent exposure of 9%, and the pH dependencies of the residues in the chain could be explained with the relayed imidic acid exchange mechanism, which would increase k_H in relation to k_{OH}; this would result in an increase of pH_{min} (see Eq. 2), which in turn would result in a negative pH dependence when $pH_{min} > 4.9$. The pH dependencies of the other amide protons that belong to hydrogen-bonded peptides could be explained along similar lines, given that the majority of the free carbonyls of the chains have high surface exposures (Fig. 4) and are thus readily protonated.

There is no correspondence to the very low pH_{min} that were observed for the residues at the N-termini of the

TABLE 2 Summary of structural data (Williamson et al., 1985) and experimental exchange rates of the amide protons in BUSI IIA (Wüthrich et al., 1984)

	$lg(k_{ex})$ (k_{ex} in s^{-1})		$\Delta lg(k_{ex})/\Delta pH$	d_{HWM}		% a.s.a.	
Residue	pH 4.0	pH 4.9		N	O*	N	O*
Ala8	−3.8	−3.3	0.5	3.3	4.9	0.9	0.0
Glu9	>−2.8	>−2.8		3.6	3.0	0.0	7.3
Phe10	−3.8	−3.6	0.2	4.0	2.9	0.0	31.0
Lys11	−3.6	>−2.8	>0.9	3.4	3.1	0.0	0.0
His24	−3.5	−3.9	−0.4	5.6	4.0	0.0	0.0
Cys25	−3.5	−3.8	−0.3	7.0	6.7	0.0	0.0
Gly26	−3.8	−4.3	−0.6	5.0	5.0	0.0	0.0
Ser27	−3.6	−3.8	−0.2	4.2	3.0	0.0	0.0
Thr31	−3.2	>−2.8	>0.4	3.3	4.2	0.4	0.0
Tyr32	−4.5	−4.7	−0.2	4.4	2.8	0.0	23.3
Gly33	>−2.8	>−2.8		3.0	4.5	20.4	0.0
Lys35	>−2.8	>−2.8		5.6	4.8	0.0	0.0
Cys36	−4.0	−3.7	0.4	5.2	5.0	0.0	0.0
Ala37	−4.6	−5.1‡	−0.4	3.7	4.0	0.0	0.0
Phe38	−5.3	−5.8‡	−0.4	4.9	3.3	0.0	3.0
Cys39	−5.0	−5.5‡	−0.4	5.3	3.8	0.0	0.0
Lys40	−4.9	−5.4‡	−0.4	3.7	2.9	0.0	0.8
Ala41	−5.1	−5.6‡	−0.4	3.6	2.8	0.0	5.7
Val42	−5.6	−6.0‡	−0.3	3.3	3.0	0.0	0.0
Met43	−4.5	−4.3	0.3	3.9	2.8	0.0	7.1
Lys44	−4.3	−4.0	0.3	4.0	2.8	0.0	36.0
Leu51	>−2.8	>−2.8		3.8	4.9	0.0	0.0
Lys52	−4.0	−4.4	−0.5	4.3	3.0	0.0	8.8
Arg54	>−2.8	>−2.8		2.9	4.9	5.3	0.0
Gly55	−3.9	−3.4	0.5	3.6	2.9	0.1	25.0

Only residues participating in chains of hydrogen-bonded peptides are tabulated.

* The carbonyl oxygen of the same peptide, i.e. of the preceding residue.

‡ Measured at pH 5.2.

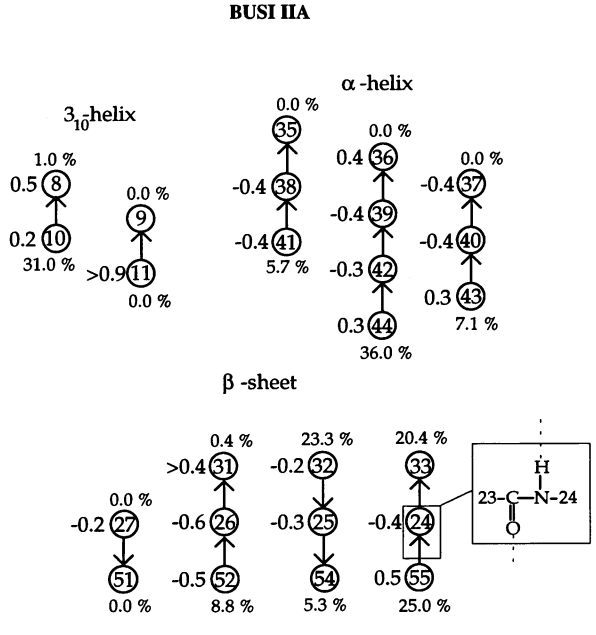


FIGURE 4 Residues participating in chains of peptide hydrogen bonds in BUSI IIA (Williamson et al., 1985). The $\Delta lg(k_{ex})/\Delta pH$ values are shown beside the residues. Other numbers/symbols are the same as in Fig. 3.

helices in BPTI (Fig. 3) to residues with the same locations in BUSI IIA (Fig. 4), since that would have been seen as strongly positive pH dependencies at the N-terminal of the helix. The relatively high d_{HWM} for the amide groups of Lys35, Cys36, and Ala37 (5.6, 5.2, and 3.7 Å, respectively) show that this end is rather inaccessible to the solvent. A deprotonation of these amides by hydroxide ions would be less probable than for the more exposed N-terminal in BPTI. We therefore expect a less pronounced decrease of pH_{min} for these residues in BUSI IIA and consequently a less positive (negative for Ala37) pH dependence.

Calbindin D_{9K}

The estimated $\Delta lg(k_{ex})/\Delta pH$ from k_{ex} measurements at pH 6.0 (Skelton et al., 1992b) and at pH 7.25 (Linse et al., 1990) for calbindin D_{9K} are rather crude because the exchange rates at the different pH values were measured with different techniques. Moreover, the method used at pH 7.25 leads to larger uncertainties (Skelton et al., 1992b) and was performed on the wild type, with a mixture of *cis*- and *trans*-Pro43 that was mutated to Gly43 in the measurements at pH 6.0. The higher pH range of the measured exchange rates compared with BUSI IIA (see above) would also result in a less relative contribution of the relayed imidic exchange mechanism compared to the base-catalyzed exchange mechanism. $\Delta lg(k_{ex})/\Delta pH$ was negative for only 25% (16 of 63) of the amide protons for which it could be estimated. The strong electrostatic fields that are generated by the calcium ions will also introduce difficulties in revealing correlations between the location of an amide in the protein and the pH dependence of its exchange rate. Nevertheless, we can see some features in the pH dependence of peptides forming hydrogen-bonded chains in calbindin D_{9K} (Fig. 5) that point toward an existence of a mechanism such as in Fig. 1.

Thirteen of the total 16 residues with a negative pH dependence in calbindin D_{9K} are located in chains of peptide hydrogen bonds. The carbonyl group of Ala14 coordinates the Ca^{2+} ion in calcium loop I (Ala14-Gln27; Szebenyi and Moffat, 1986), and the last three residues of this chain, terminated by the non-hydrogen-bonded Ala14-O (Ile9, Lys12, and Ala15; see Fig. 5) have $\Delta lg(k_{ex})/\Delta pH$ values close to 1 (Fig. 5). This might be understood given that the presence of the Ca^{2+} ion at the carbonyl oxygen of Ala14 produces an acidic amide proton of Ala15 through its electron withdrawing effect, and the proton is exchanged through the base-catalyzed mechanism. The electrostatic field from the ion would propagate through the chain because of the π-bond cooperativity along the chain of hydrogen-bonded peptides (Jeffrey and Saenger, 1991) and acidify all amide protons belonging to this chain of peptide hydrogen bonds. The calcium ion will also repel all nearby protons in the solution and thus effectively inhibit protonation of Ala14-O. The increased acidity may possibly also lead to a reversal of the relayed imidic acid exchange mechanism, in which case the exchange of amide protons

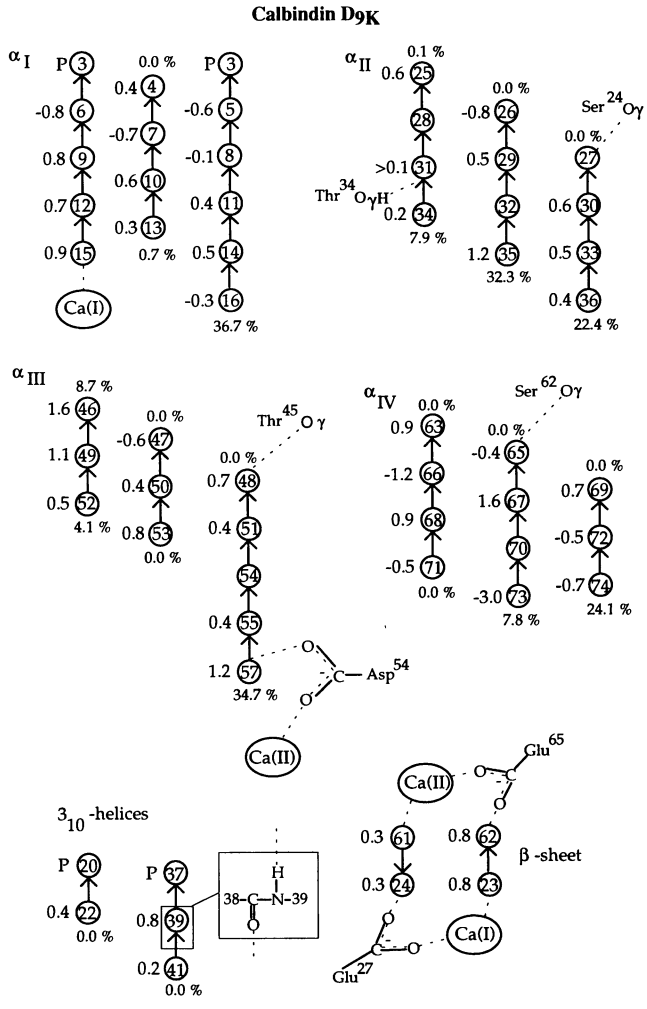


FIGURE 5 Residues participating in chains of peptide hydrogen bonds in calbindin D_{9K} (Kördel et al., 1993). The $\Delta lg(k_{ex})/\Delta pH$ values are shown beside the resides. Other numbers/symbols the same as in Fig. 3. The numbering of the calcium ions (I and II) refers to their calcium binding sites (Szebenyi and Moffat, 1986).

along the chain would be initiated by a deprotonation of Ile9-NH (Fig. 5). The deprotonation of the next residue in the chain, Leu6-NH, by hydroxide ions will have a low probability because of its high d_{HWM} (5.3 Å compared with 3.6 Å for Ile9; see Table 3). The negative pH dependence of the exchange rate for this residue would then be a result of an increased dominance of the relayed imidic acid exchange mechanism (Fig. 1) or by the imidic acid mechanism, increasing pH_{min} according to Eq. 2.

Many of the free amide protons (at the N-termini) in a chain of peptide hydrogen bonds in the α-helices show positive pH dependencies of the exchange rates (Fig. 5). This is consistent with the very low pH_{min} values found at the N-termini of the helices in BPTI (Fig. 3), and the explanation for the strongly positive pH dependence at the N-termini would then be the same as for BPTI, i.e., parallel dipoles that acidify the N-terminal protons and often a low d_{HWM} for the carbonyl oxygens of these peptides (Table 3).

TABLE 3 Summary of structural data (Kördel et al., 1993; Svensson et al., 1992) and experimental exchange rates (Skelton et al., 1992b; Linse et al., 1990) of the amide protons in calbindin D_{9K}

Residue	$1g(k_{ex})$ (k_{ex} in s^{-1}) pH 6.0*	pH 7.25‡	$\Delta 1g(k_{ex})/\Delta pH$	d_{HWM} N	d_{HWM} O§	% a.s.a. N	% a.s.a. O§
Glu4	−0.35	0.20	0.44	3.0	3.0	0.0	0.0
Glu5	−0.75	−1.50	−0.60	3.9	2.9	0.0	1.0
Leu6	−1.30	−2.30	−0.80	5.3	3.6	0.0	0.0
Lys7	−1.35	−2.20	−0.68	5.0	6.2	0.0	0.0
Gly8	−1.35	−1.50	−0.12	3.2	4.0	0.4	0.0
Ile9	−2.99	−2.00	0.79	3.6	3.1	0.0	0.4
Phe10	−4.01	−3.30	0.57	6.2	5.6	0.0	0.0
Glu11	−3.75	−3.30	0.36	4.9	6.3	0.0	0.0
Lys12	−3.80	−2.90	0.72	3.7	4.0	0.0	0.0
Tyr13	−6.21	−5.80	0.33	5.4	3.3	0.0	0.7
Ala14	−6.57	−6.00	0.46	6.6	5.0	0.0	0.0
Ala15	−2.60	−1.50	0.88	5.1	4.8	0.0	0.0
Lys16	−3.53	−3.90	−0.30	3.9	2.8	0.0	36.7
Gln22	−5.73	−5.20	0.42	5.8	4.8	0.0	0.0
Leu23	−5.14	−4.20	0.75	7.3	5.9	0.0	0.0
Ser24	−7.15	−6.70	0.36	5.2	6.2	0.0	0.0
Lys25	−1.15	−0.40	0.60	3.4	5.4	0.1	0.0
Glu26	−0.48	−1.50	−0.82	3.5	4.4	0.0	0.0
Glu27	−4.54			5.4	3.7	0.0	0.0
Leu28	<−7.40	<−8.20		6.9	5.8	0.0	0.0
Lys29	−6.13	−5.50	0.50	5.6	7.2	0.0	0.0
Leu30	−5.10	−4.30	0.64	4.3	4.9	0.0	0.0
Leu31	<−7.40	−7.30	>0.08	5.3	3.4	0.0	0.0
Leu32	<−7.40	−7.90		6.8	6.2	0.0	0.0
Gln33	−5.38	−4.80	0.46	5.4	5.8	0.0	0.0
Thr34	−3.42	−3.20	0.18	3.7	3.2	0.0	7.9
Glu35	−3.56	−2.00	1.25	4.4	2.9	0.0	32.3
Phe36	−4.31	−3.80	0.41	4.5	2.9	0.0	22.4
Leu39	−1.35	−0.30	0.84	3.6	3.3	0.0	1.1
Lys41	−1.10	−0.80	0.24	4.3	3.0	0.0	0.0
Leu46	−1.15	0.80	1.56	3.0	3.7	8.7	0.0
Asp47	−1.23	−2.00	−0.62	4.6	5.4	0.0	0.0
Glu48	−1.23	−0.30	0.74	4.0	4.3	0.0	0.0
Leu49	−2.86	−1.50	1.09	3.6	3.3	0.0	1.0
Phe50	−3.76	−3.20	0.45	6.0	5.0	0.0	0.0
Glu51	−4.30	−3.80	0.40	4.7	5.0	0.0	0.0
Glu52	−3.99	−3.40	0.47	3.2	3.0	0.7	4.1
Leu53	−5.06	−4.00	0.85	5.5	3.7	0.0	0.0
Asp54	−4.59			5.4	5.6	0.0	0.0
Lys55	−4.45	−3.90	0.44	4.1	2.8	0.0	3.0
Gly57	−1.95	−0.50	1.16	3.7	2.8	0.0	34.7
Val61	−5.89	−5.50	0.31	6.9	5.5	0.0	0.0
Ser62	−6.08	−5.10	0.78	5.0	7.1	0.0	0.0
Phe63	−1.10	0.00	0.88	4.3	5.7	0.0	0.0
Glu65	−4.04	−4.50	−0.37	4.2	3.1	0.0	0.6
Phe66	−6.40	−7.90	−1.20	5.4	4.2	0.0	0.0
Gln67	−4.03	−2.00	1.62	4.9	5.9	0.0	0.0
Val68	−2.60	−1.50	0.88	3.6	3.0	0.0	0.1
Leu69	−4.89	−4.00	0.71	5.9	5.2	0.0	0.0
Val70	−4.10			5.4	4.8	0.0	0.0
Lys71	−1.35	−2.00	−0.52	3.4	4.1	0.0	0.0
Lys72	−1.35	−2.00	−0.52	4.6	4.7	0.0	0.0
Ile73	−1.35	−5.10	−3.00	3.6	3.0	0.0	7.8
Ser74	−1.15	−2.00	−0.68	3.8	2.9	0.0	24.1

The wrongly assigned amide protons at pH 7.25 have been corrected according to Skelton et al. (1992b). Only residues participating in chains of hydrogen bonded peptides (excluding prolines) are tabulated.

*Skelton et al., 1992b; ‡ Linse et al., 1990.

§ The carbonyl oxygen of the same peptide bond, i.e., of the preceding residue.

It is worth noting that the exchange rates of amide protons in the chain of hydrogen-bonded peptides, 46–49-52 (see Fig. 5), which is close to the protein-solvent interface, have positive pH dependencies, whereas more buried chains such as 4–7-10–13, 26–29-32–35, and 47–50-53 (see Table 3) have a higher content of amide protons with a negative pH dependence of the exchange rate. This might be an indication that the less accessible the peptides are to the solvent, the more important is the relayed imidic acid exchange mechanism compared with the base-catalyzed mechanism. pH_{min} will increase (Eq. 2), resulting in a negative pH dependence of the exchange rate if k_H/k_{OH} is large enough. The chain 3–6-9–12-15 is also very close to the protein-solvent interface (Table 3) and is dominated by residues that have positive pH dependencies of the exchange rate. It is plausible that both the presence of the calcium ion at Ala14-O, blocking protonation of this oxygen and hence the action of the relayed imidic acid exchange mechanism, and the surface location of the chain are responsible for the positive $\Delta lg(k_{ex})/\Delta pH$ values for these residues.

The positive $\Delta lg(k_{ex})/\Delta pH$ values in the β-sheet (Fig. 5) is probably also an effect of the calcium ions very close to both the chain-terminal carbonyls. The ions are blocking the protonation of these carbonyls, and the exchange rates would be raised by increasing the pH. Interestingly, when cadmium is bound to only site II of calbindin D_{9K}, the exchange rate of Ser24 becomes too rapid for the ^{15}N chemical shift of this residue to be determined (Akke et al., 1991; Skelton et al., 1992a,b). This indicates the existence of imidic acid intermediates in this chain, because with only site II occupied, the electron withdrawing effect from the cadmium ion would propagate along the chain and considerably acidify the amide group of Ser24, making it readily exchangeable. Upon binding of the second ion to site I the whole hydrogen bonding network between the ions, including the side chain of Glu27 (Fig. 5), should be strengthened and thus should considerably decrease the exchange rate of Ser24.

SUMMARY AND CONCLUDING REMARKS

Upon inspection of the variation in $lg(k_{min})$, $lg(k_{OH})$, and $lg(k_H)$ (Tüchsen and Woodward, 1985a) with $d_{HWM}(O)$ of the peptide carbonyl oxygen for the solvent-exposed amide protons in BPTI, we find that the exchange rates (k_{min}) are in general slower for amides with more buried carbonyls. The variation of $lg(k_{OH})$ with $d_{HWM}(O)$ for these residues revealed that residues with buried peptide carbonyls belonging to the β-sheet, had about 10–100 times lower k_{OH} values than free peptides. We can think of two possible reasons for this decrease in k_{OH}. The first explanation concerns the relative probabilities of O-protonation ($pK_a \sim 0$), which is more probable than a "direct" N-deprotonation ($pK_a \sim 18$) for pH values less than ~9. This pH value is well above pH_{min}, where the exchange occurs according to the base-catalyzed mechanism, i.e., a hindered protonation

of the carbonyl oxygen would decrease k_{OH}. Secondly, it can be an effect of the fact that the partial negative charge on the oxygen in the imidate ion intermediate (Eq. 3) must be solvated; this is unfavorable if that oxygen is buried in a less polar environment, which thus would lower k_{OH}. For amide protons in the N-terminal helices, k_{OH} is instead generally increased relative to a free peptide. This could be explained by the parallel chains of hydrogen-bonded peptides in helices, which further acidifies the N-terminal amide protons and diminishes the importance for k_{OH} of a solvation of the oxygen in the imidate ion intermediate or a prior protonation of the carbonyl oxygen.

For some residues in the central β-sheet that have low k_{OH} values, a corresponding lowering of the k_H value was not observed. A lower k_H value would be expected if the imidic acid mechanism is invoked, considering that the peptide carbonyls of these residues are deeply buried in the protein interior. The less pronounced decrease in k_H, seen as an unusually high pH_{min} of these residues, might be explained by the action of the relayed imidic acid exchange mechanism. The free carbonyls at the other end of the chain of peptide hydrogen bonds are more acidic than in a free peptide because of constructively interacting dipoles and the π-bond cooperativity (Jeffrey and Saenger, 1991). They are also often highly solvent exposed and thus readily protonated. The relative probabilities of O-protonation/N-deprotonation (see above) are expected to be maintained for the free carbonyl oxygen at one end of the chain and for the free amide at the other end in a chain of peptide hydrogen bonds. Assuming the existence of the relayed imidic acid exchange mechanism (Fig. 1), we therefore expect that it is essentially acid-catalyzed and its action thus accelerates k_H more than k_{OH}, shifting pH_{min} upward. This effect would provide an explanation for the high pH_{min} values of the residues with exposed amides of the central β-sheet in BPTI. The relayed imidic acid exchange mechanism would then also explain the observed successive increase of the pH_{min} along a chain of hydrogen-bonded peptides in the direction from the free amide to the free carbonyl, observed in BPTI. The contribution of the relayed imidic acid exchange mechanism is expected to be larger for amides closer to the free carbonyl group, because fewer high energy imidic acid intermediates are involved (see Fig. 1). k_H/k_{OH} would increase in the same direction and thus successively increase pH_{min} (Eq. 2).

The activity of the relayed imidic acid exchange mechanism will depend on many factors such as the pH of the solution, the stability and solvent accessibility of the peptide hydrogen bonds in the chains, and the solvent accessibility of the free carbonyl oxygens. Since the relayed imidic acid exchange mechanism would essentially be acid catalyzed it should be more active in more acidic solutions, as also was inferred by comparing BUSI IIA (pH range 4–5) with calbindin D_{9K} (pH range 6–7), for which 45 and 25% of all residues, respectively, have negative $\Delta lg(k_{ex})/\Delta pH$ values. The majority of the free peptides, i.e., non-hydrogen-bonded peptide amides and carbonyls, showed a positive pH dependence, which is consistent with the fact that the base-catalyzed mechanism dominates for these residues.

In BUSI IIA and calbindin D_{9K} we noted that the majority of the residues with negative $\Delta lg(k_{ex})/\Delta pH$ values were found in chains of peptide hydrogen bonds. This might be due to the action of the relayed imidic acid exchange mechanism, because an increase of k_H/k_{OH} and consequently of pH_{min} would result in a negative pH dependence if the pH range (4–5 for BUSI IIA and 6–7 for calbindin D_{9K}) is less than pH_{min}. An inhibition of the relayed imidic acid exchange mechanism was observed in calbindin D_{9K} as pH dependencies close to 1 for amide protons belonging to chains of peptide hydrogen bonds for which the free carbonyls coordinate a calcium ion. The presence of the calcium ion inhibits a protonation of the free carbonyl oxygen and thus the action of the relayed imidic acid exchange mechanism. However, the strong electrostatic field from the ion propagates through the peptides, which further acidifies the free amide at the other end of the chain, and a reversal of the mechanism might also be feasible. The free carbonyl groups in the β-sheet are also coordinating calcium in calbindin D_{9K}, and they also have pH dependencies close to 1.

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