Kinetics of Local Helix Formation in Poly-L-Glutamic Acid Studied by Time-Resolved Photoacoustics: Neutralization Reactions of Carboxylates in Aqueous Solutions and Their Relevance to the Problem of Protein Folding

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ABSTRACT Photoactivatable caged protons have been used to trigger proton transfer reactions in aqueous solutions of acetate, glutamate, and poly-L-glutamic acid, and the volumetric and enthalpic changes have been detected and characterized by means of time-resolved photoacoustics. Neutralization of carboxylates in aqueous solutions invariably results in an expansion of the solution due to the disappearance of two charges and is accompanied by little enthalpic change. The reactions occur with thermally activated, apparent bimolecular rates on the order of 10^{10} $M^{-1}s^{-1}$. In the case of aqueous solutions of poly-L-glutamic acid at pH around the pK_a of the coil-to-helix transition, diffusional binding of a proton by carboxylates is followed by a sequential reaction with rate 1.06 ($\pm$ 0.05) $\times$ $10^7 s^{-1}$. This step is not thermally activated in the temperature range we have investigated and is likely related to local formation of hydrogen bonds near the protonation site. This structural event may constitute a rate-limiting step in helix propagation.

INTRODUCTION

The structure of proteins is influenced to a large extent by the charge state of ionizable groups on the side chains of several amino acids (Creighton, 1990). At pH below neutrality, the amino acids mainly involved in the ionic equilibria are glutamic acid (glu) and aspartic acid (asp), with carboxylate pK_a of 4.33 (at 5°C) and 4.01 (at 1°C), respectively (Weast, 1968). Besides these groups, histidines play an important role in many cases of interest, because their pK_a, normally around 6 (Weast, 1968), may be strongly shifted in either direction due to interactions with nearby residues, as in the case of apomyoglobin (Hughson et al., 1991; Cocco et al., 1992).

Techniques based on rapid mixing to generate a pH jump have been used traditionally to study the dynamics of a protein response to acidification of the solution, but the early protonation steps (times $<$ 1 ms) were out of reach. Only with the introduction of laser-based techniques have the faster events become accessible (Gutman and Nachliel, 1990).

In this work we use a nanosecond UV laser and photolabile caged protons to generate a pH jump and monitor proton binding to carboxylates of the model compounds acetate (pK_a = 4.77 at 5°C), glutamate (pK_a^1 = 2.19 and pK_a^2 = 4.33 at 5°C), and poly-L-glutamic acid (PLG). Time-resolved photoacoustics was used to monitor the structural response of the systems (Braslavsky and Heibel, 1992). We have recently applied this experimental methodology to proton transfer reactions in aqueous solutions, characterizing the solvation of photoinduced charges (Bonetti et al., 1997; Viappiani et al., 1998; Losi and Viappiani, 1998), the formation of water molecules from proton and hydroxide (Viappiani et al., 1998; Bonetti et al., 1997) and the reaction of protons with poly-L-lysine (Viappiani et al., 1998) and apomyoglobin (Abbruzzetti et al., 2000).

Volume changes in proton transfer reactions arise from electrostrictive effects due to the changes in the net number of charges present in solution and to the alterations in the specific interactions between solute and solvent molecules (Van Eldick et al., 1989). When proton transfer reactions induce conformational changes in proteins, additional volume changes may result from structural rearrangements of the macromolecules.

Our aim here is to investigate the structural response of carboxylate model systems and polypeptides due to protonation and their relation to early events in acid-induced protein folding/unfolding. Despite the fact that PLG sequences are not found in naturally occurring polypeptides, PLG nevertheless represents an interesting system for model studies because it undergoes a pH-dependent random-coil-to-helix transition, with an apparent pK_a of 5.4 (vide infra). Neutralization of γ carboxylates occurs randomly on the polypeptide chain and reduces the intramolecular electrostatic repulsive forces. At a sufficient level of protonation, the decreased repulsion permits conformational changes stabilized by the local formation of hydrogen bonds in PLG. This local formation of secondary structure may represent the ultimate rate limiting step in helix formation for these model polypeptides.

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MATERIALS AND METHODS

Chemicals

Bromocresol purple (Kodak) or Brilliant Black BN (Aldrich) (Abbruzzetti et al., 1999) in water were used as photocalorimetric references in the photoacoustic experiments. The pH of the reference solution was adjusted to 9 for bromocresol purple and to 6 for Brilliant Black BN in order to avoid instability in optical absorption at the excitation wavelength.

PLG (MW = 17, 500, corresponding to 82 glu residues), L-glutamic acid (glu), sodium acetate and *o*-nitrobenzaldehyde (*o*NBA) were obtained from Sigma. *o*NBA was recrystallized from ethanol before use.

The pH of the solutions was adjusted by the addition of concentrated NaOH or HCl. The samples were nitrogen-saturated to decrease potential buffering effects due to the presence of dissolved CO_2.

Steady-state absorption was measured with a Jasco 7850 UV-vis spectrophotometer. Far-UV circular dichroism was measured with a Jasco J700. All instruments were equipped with temperature-controlled sample holders.

Depending on the experiment, the concentrations of glutamate and acetate were 1 to 300 μM, whereas the concentration of PLG was 0.1 to 50 μM (residue concentrations, 8.2 to 4100 μM). The concentrations at which thermodynamic parameters were determined are reported in the footnotes of the tables in the results section (vide infra).

Photoacoustic setup

The experimental setup has been described previously (Murgida et al., 1998; Pelagatti et al., 1998; Abbruzzetti et al., 2000). Photoexcitation was achieved by the third harmonic (λ = 355 nm) of a nanosecond, Q-switched Nd:YAG laser (Surelite II-10, Continuum, Santa Clara, CA) operating at 1 Hz. The unfocused beam was attenuated and shaped by a slit (280 μm width) positioned near the cuvette. The pressure wave induced in solution was detected by a PZT piezoelectric transducer (Panametrics V-103). The signal was then amplified (60 db) and recorded by a digitizing oscilloscope (LeCroy 9450A) operated at 2.5 ns/channel. A quartz cuvette was mounted inside a temperature controlled sample holder (TASC 300; Quantum Northwest, Inc., Spokane, WA) and degassed with nitrogen. Data acquisition and analysis were performed by means of dedicated software (Sound Acquisition and Sound Analysis, Quantum Northwest, Inc.). The number of laser shots averaged to generate each sample waveform was 9, whereas 100 laser shots were averaged to generate each reference waveform.

Analysis of photoacoustics data

The principles of deconvolution of photoacoustics waveforms have been described (Small et al., 1992; Small, 1992; Rudzki et al., 1985). The sample waveform is assumed to be convolution of the reference waveform and a sum of exponential decay functions:

$$H(t) = \sum_i \frac{\varphi_i}{\tau_i} e^{-t/\tau_i} \quad (1)$$

where φ_i is the preexponential factor of the transient with lifetime τ_i. The values of φ_i and τ_i are the results of the deconvolution analysis.

We have determined the structural volume changes as a function of the concentration of the acceptor molecule using a two-temperature method (Gensch and Braslavsky, 1997). The sample waveform was acquired at $T_{\beta=0}$ = 3.9°C (Weast, 1971) and compared to a reference waveform acquired at a slightly higher temperature, $T_{\beta\neq0}$ = 6.0°C (since a reference waveform cannot be measured at $T_{\beta=0}$). The sample waveforms measured at 3.9°C originate solely from structural changes in the solution and include no enthalpic contribution.

The extent of the observed structural volume change for each decay component, ΔV_i, is calculated from φ_i as:

$$\Delta V_i = \varphi_i E_\lambda \left(\frac{\beta}{C_p\rho}\right)_{\beta\neq0} \quad (2)$$

where E_λ is the molar energy content of the laser pulse, $(\beta/C_p\rho)_{\beta\neq0}$ is the thermoelastic parameter of the solution at $T_{\beta\neq0}$, β is the thermal expansion coefficient, C_p is the specific heat, and ρ is the density. For dilute aqueous solutions and for pH values above 4 the parameter $(C_p\rho/\beta)$ has essentially the same value it has for water at pH = 7 and can be determined from literature values (Weast, 1971).

Experiments conducted at multiple temperatures between 5 and 50°C have been used to determine for each step the heat release, the volume change, and, from the temperature dependence of the rate constants, the activation energy (Callis et al., 1972; Peters and Snyder, 1988; Braslavsky and Heibel, 1992). For this multiple-temperature method, deconvolution is performed for each reference/sample pair and the energy content of the transients, $E_\lambda\varphi_i$, at each temperature is calculated and plotted versus the temperature-dependent parameter $(C_p\rho/\beta)$. From the linear relation

$$\varphi_i E_\lambda = Q_i + \Delta V_i \left(\frac{C_p\rho}{\beta}\right) \quad (3)$$

one can obtain Q_i (the heat released per mole photons absorbed) and ΔV_i (the structural volume change per mole photons absorbed) for each of the steps.

If the quantum yield for release of protons, Φ_{H^+}, is known, from Eqs. 2 and 3 it is possible to determine the molar reaction volume $\Delta V_{R,i} = \Delta V_i/\Phi_{H^+}$.

The temperature dependence of the rate constants has been analyzed using the relationship

$$k_2 = k_2^0 e^{-E_a/RT} \quad (4)$$

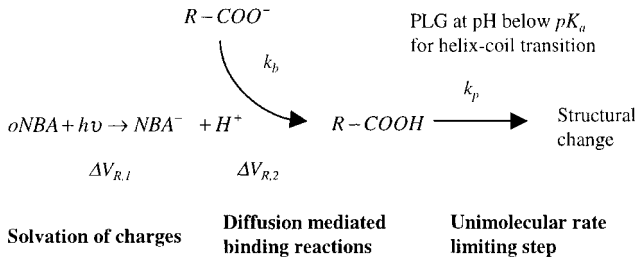
where $k_2 = 1/\tau_2$ (vide infra). From the linear plot of $ln(k_2)$ vs. $1/T$ (Arrhenius plot), it is possible to extract the activation energy E_a and the frequency factor $k_2{}^0$.

RESULTS

In our experiments, the proton concentration is rapidly increased using a nanosecond UV laser pulse to photolyze *o*NBA (the caged proton). The photolysis is irreversible and the pK_a of the acid intermediate formed upon photoexcitation of *o*NBA is 2.1 (Pelagatti et al., 1998). This renders the rate and the quantum yield for photoinduced proton release, Φ_{H^+}, independent of the pre-pulse pH, at pH values above 4. If the pH of the solution is not lowered below 4, the nitrosobenzoic acid (the final product of *o*NBA photolysis) is completely dissociated and proton release by *o*NBA is irreversible. In all of our experiments, the concentration of photoreleased protons in the excitation volume (~20 μl) is approximately 1 μM for each flash, independent of the pre-pulse pH. The magnitude of the pH jump depends on the pre-pulse pH, but proton transfer reactions are induced in the whole pH range we have investigated (Abbruzzetti et al., 2000). The protons, which are released within 10 ns, then react with proton acceptors through diffusion mediated processes and, due to the large excess of acceptors (not only in the excitation volume but also in the surrounding solution),

the pH returns to the pre-pulse value before the next laser flash (1 s). The large excess of proton acceptor residues also assures that, at most, 1 proton is bound to the small percentage of PLG molecules that participate in protonation reactions after each flash.

We have conducted experiments to determine the volumetric response of the proton acceptors (acetate, glu, and PLG) as a function of the pre-pulse pH, the concentration of acceptors, and the temperature. The ground state protonation reactions induced by the laser pH-jump are diagrammed in Scheme 1, where the parameters relevant to our study are also reported.



Scheme 1.

Concentration-dependent experiments: two-temperature method

o*NBA in water*

The release of protons by *o*NBA, both in the presence and in the absence of other solutes, was accompanied by a fast contraction of the solution (lifetime <10 ns) for all of the experiments we performed, in accordance with the previously published data (Pelagatti et al., 1998; Bonetti et al., 1997; Viappiani et al., 1998). We have estimated the molar reaction volume for the fast contraction as an average of results for low acceptor concentration (<20 μM for PLG, <200 μM for acetate and glutamate). The values obtained are reported as $\Delta V_{R,1}$ (reaction volume change per mole of added proton for the first decay component) in Table 1. Although the $\Delta V_{R,1}$ values are unlikely to involve the acceptors, the amplitude of this contraction appears to depend significantly on the nature of the acceptor. This observation may arise partly from the influence of the protonatable solutes on specific interactions between *o*NBA or the products and the solvent. A similar finding has been recently reported (Borsarelli and Braslavsky, 1998) for the photoinduced formation of the metal-to-ligand charge-transfer state in ruthenium(II) bipyridine cyano complexes in the presence of different salts. The authors were able to explain the salt dependence of the photoinduced volume changes with the effect of these chaotropic and organizing agents on the extended three-dimensional network of hydrogen bonds in water.

o*NBA with aqueous glutamate or acetate*

When protons are released in the presence of either glu or acetate at neutral pH, a volumetric expansion, with lifetime dependent on the concentration of the acceptor, follows the fast contraction. Fig. 1 illustrates the waveform shapes obtained for the reference compound at T = 6.0°C and for aqueous solutions containing *o*NBA and three concentrations of acetate at pH = 7 and 3.9°C. Similar waveforms were obtained for the protonation of glu and for PLG at pH 7.4. The deconvolution analysis in each case indicated a biexponential decay with the expected fast ($\tau_1 < 10$ ns) contraction followed by a slower expansion characterized by a lifetime, τ_2, which changed inversely with the concentration of acceptor. Because the concentration of photoreleased protons (below 1 μM) was much lower than the concentration of acceptors, pseudo-first-order kinetics can be expected for the protonation reaction. From the slope of a plot of rate constant, $k_2 = 1/\tau_2$, as a function of the acceptor concentration (illustrated in Fig. 3 for PLG at pH 7.4, filled symbols; vide infra), we obtained bimolecular rate constants, k_b, as reported in Table 1. The reaction volumes, $\Delta V_{R,2}$, reported in Fig. 2 were determined from the preexponential factors, φ_2, using Eq. 2 and dividing each ΔV_2 by the deprotonation quantum yield for *o*NBA, $\Phi_{H^+} = 0.4$ (George and Scaiano, 1980).

TABLE 1 Structural volume changes and proton binding rate constants determined using the two-temperature method

Proton acceptor	$\Delta V_{R,1}$ (ml/mol)	$\Delta V^0_{R,2}$ (ml/mol)	k_b ($10^{10}M^{-1}s^{-1}$)	k_p (s^{-1})
Acetate	−9.2 ± 0.4*	8.9 ± 0.3	5.8 ± 0.2	
Glu	−8.5 ± 0.2	6.4 ± 0.5	5.9 ± 0.4	
PLG (pH 7.4)	−9.5 ± 0.5	13 ± 2	165 ± 7†	
PLG (pH 5)	−7.3 ± 0.2‡	7.2 ± 0.9§	61 ± 4†‡	1.06 ± (0.05)×10^7§

$\Delta V_{R,1}$ is the structural volume change for deprotonation of *o*-nitrobenzaldehyde (release and solvation of the caged proton). $\Delta V^0_{R,2}$ is the structural volume change for protonation of the acceptor. Values of k_b are the calculated bimolecular rate constants for binding of protons to the target; k_p is the rate-limiting constant observed at high PLG concentrations.

*Uncertainty values were estimated as the standard deviations for three separate determinations of the parameter.

†This result is based on polymer concentration.

‡Data from experiments at [PLG] < 2 μM, where cross-correlation between fitting parameters is negligible.

§Data from experiments at [PLG] > 20 μM, where the residual unimolecular reaction is better characterized (see text).

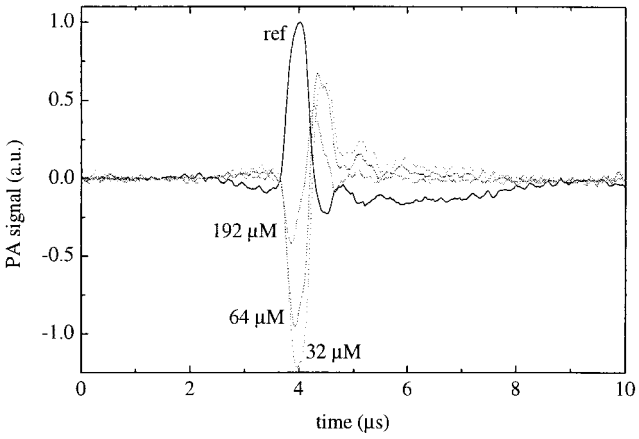


FIGURE 1 Photoacoustic signals of an aqueous solution containing *o*NBA and acetate at pH = 7 as a function of acetate concentration as indicated at $T_{\beta=0}$ = 3.9°C. The decrease in waveform amplitude with increasing acetate concentration derives from summing the signals due to freeing the caged protons with that (of opposite amplitude) due to binding the protons to acetate. The reference compound was bromocresol purple (*solid line*), measured at $T_{\beta\neq0}$ = 6.0°C.

Because the concentration of $RCOO^-$ (>10 μM) is large relative to the increase in proton concentration (~1 μM), then the relaxation of reaction (5)

$$RCOO^- + H^+ \underset{k_d}{\overset{k_a}{\rightleftarrows}} RCOOH \tag{5}$$

to the new equilibrium is expected to lead to an exponential decay characterized by an apparent rate constant $k = k_a[RCOO^-] + k_d$ (Laidler, 1987). Not all of the added protons bind to acceptor (i.e., the proton concentration after reequilibration is higher than that before the proton pulse). For low acceptor concentrations the increase in proton concentration represents a significant fraction of the added protons and the measured $\Delta V_{R,2}$ (volume change per mole of added protons) will be low. At high acceptor concentrations the fraction of unreacted protons is small and $\Delta V_{R,2}$ reaches a plateau value, $\Delta V^0_{R,2}$. Again assuming that acceptor is greatly in excess of the added protons, the dependence of $\Delta V_{R,2}$ on acceptor concentration is described (Laidler, 1987) by the following equation:

$$\Delta V_{R,2} = \Delta V^0_{R,2} \frac{k_a[RCOO^-]}{k_a[RCOO^-] + k_d} \tag{6}$$

Fig. 2 shows representative experimental results with curve fits based on Eq. 6. The recovered parameters, $\Delta V^0_{R,2}$, are reported in Table 1.

Note that, before the next laser pulse, the proton concentration in the excitation volume returns to the pre-pulse value due to diffusion between the very small excitation volume (<0.03 ml) and the much larger surrounding volume (~3 ml).

The smallest resolvable decay times (largest rate constants, highest concentration of acceptor) obtained were on the order of the time resolution of the experimental setup (about 20 ns). Increasing the concentration further led to a single, prompt volume change, which represents a sum of the fast contraction due to proton photodetachment and the subsequent binding by the carboxylates.

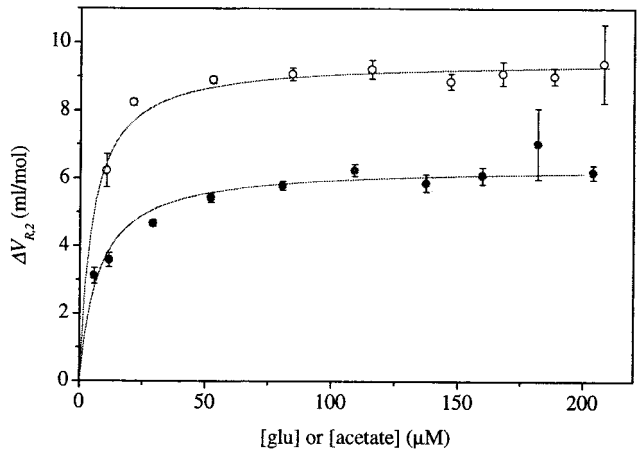


FIGURE 2 Dependence of $\Delta V_{R,2}$ on the concentration of proton acceptor for acetate (*open circles*) and glutamate (*filled circles*) at pH = 7. Fitting of the data with Eq. 6 led to the reaction volumes $\Delta V^0_{R,2}$, reported in Table 1.

*o*NBA with aqueous PLG

The results obtained for PLG at pre-pulse pH = 7.4 were very similar to the results outlined so far for glu and acetate. The rate constant for the volumetric expansion, $k_2 = 1/\tau_2$, increases linearly with PLG concentration, as shown in Fig. 3.

At lower pH, PLG is known to undergo a transition to an α-helical conformation (with an apparent pK_a = 5.4 based on far UV circular dichroism measurements, data not shown). At pre-pulse pH around and below 5.4, results were substantially different. As the concentration of PLG is increased, the rate constant for binding of protons initially increases, but soon levels off to a plateau value of about 10^7 s^{-1}; this plateau value is included as k_p in Table 1 and the results for pre-pulse pH = 5 are plotted in Fig. 3. The behavior was similar in experiments done at lower pre-pulse pH values (data not shown). We estimated the contraction for deprotonation of *o*NBA, $\Delta V_{R,1}$, using results at low PLG concentration. The extent of the volume change associated with the rate-limiting step, $\Delta V^0_{R,2}$, was estimated as an average of the volume changes measured at high PLG concentrations. The results are included in Table 1.

Temperature-dependent experiments: multiple-temperature method

The multiple-temperature method allows a determination of molar volume and enthalpy changes (from the temperature

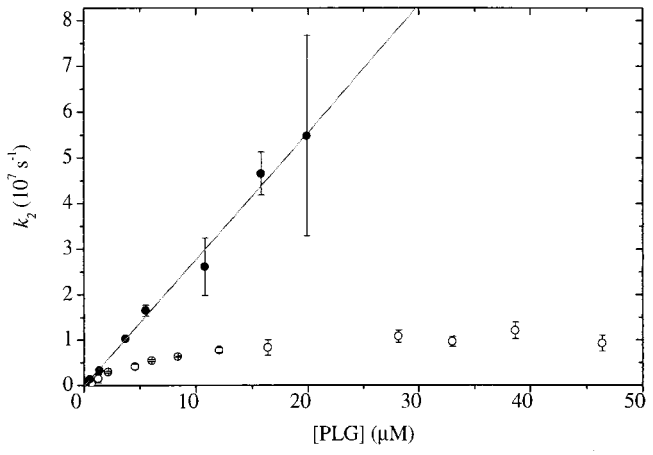


FIGURE 3 Dependence of the rate constants $k_2 = 1/\tau_2$ for the protonation of PLG at pH = 7.4 (*filled circles*) and pH = 5 (*open circles*) as a function of PLG concentration using the two-temperature method. The slope of the line shown through the data at pH = 7.4 is reported as k_b in Table 1. The rate constant measured at pH = 5 reaches a limiting value of 1.06 (± 0.05) × $10^7 s^{-1}$ above 20 μM.

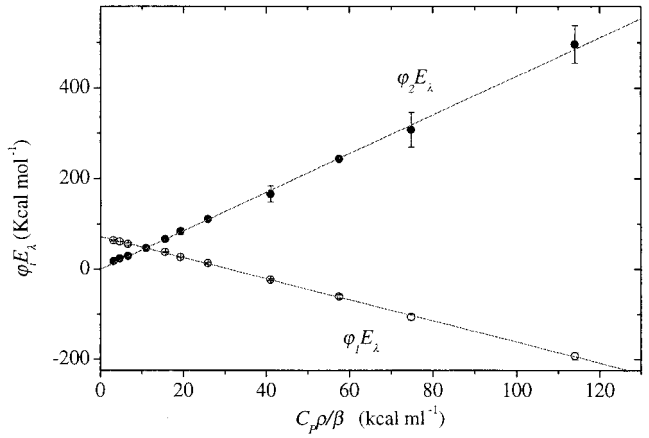


FIGURE 4 Plots of the energy content for the photodissociation of *o*-nitrobenzaldehyde ($\varphi_{1E\lambda}$) and for binding of the photo-released protons to poly-L-glutamic acid (0.8 μM; $\varphi_{2E\lambda}$) in water at pH = 7.2, as a function of the parameter $C_p\rho/\beta$. $C_p\rho/\beta$ was changed by varying the temperature between 5 and 50°C. From the linear interpolations based on Eq. 3, we obtained the volume change (from the slope) and the enthalpy change (from the intercept). The results are reported in Table 2.

dependence of the preexponential factors) and of activation energies (from the temperature dependence of the rate constants) for the neutralization reactions. At all investigated temperatures the photoacoustic signals were well described by a biexponential decay with the fast, subresolution transient due to the evolution and solvation of photodissociated ions and a slower decay, with a temperature-dependent rate constant reflecting proton transfer reactions with the acceptors present in solution.

The preexponential factors vary with temperature because the magnitude of the volume changes which result from heat deposition in the solvent are a function of temperature. Fig. 4 shows an example of the data, plotted based on Eq. 3, for results obtained at pH = 7.2 and low PLG concentration, where the binding processes are well resolved from proton release. The volume and enthalpy changes obtained from the slopes and intercepts are included in Table 2.

Fig. 5 shows examples of the Arrhenius plots for the rate of proton binding ($k_2 = 1/\tau_2$) to PLG at pH 4 using low and high polymer concentrations. The activation energies and frequency factors obtained from Arrhenius analyses of multiple-temperature data are included in Table 2.

DISCUSSION

Volume changes due to protonation reactions

It is well established that when acid is added to an aqueous solution of a protein around and below neutrality, the principal reaction is protonation of carboxylates. The expansion accompanying neutralization to carboxylic acids is affected by the nature of the group, R, attached to the carboxyl group. For instance, the volume change for neutralization of formic acid in water at 25°C is 6 ml/mol; however, it increases to 11.5 ml/mol for acetic acid, 12 ml/mol for ethanoic acid, 13.2 ml/mol for *i-Pr*COOH, and 17.1 ml/mol for *t-Bu*COOH (Van Eldick et al., 1989). Therefore, studies on simple molecules demonstrate a volume increase between 6 and 17 ml/mol, depending on the nature of R (Van Eldick et al., 1989; Rasper and Kauzmann, 1962b). If positively charged groups exist close to the carboxylate then the volume increase is only 6 or 7 ml/mol (Van Eldick et al., 1989). Reaction (5) in proteins leads generally to volume changes of about 11 ml/mol, but in some cases smaller values can be observed, possibly related to the nearness of histidine, lysine or arginine residues (Rasper and Kauzmann, 1962a).

The volume change for neutralization of glu residues in PLG at neutral pH (Tables 1 and 2) is very similar to the values reported by Kauzman for the corresponding reaction in proteins (Rasper and Kauzmann, 1962a).

Despite the expected equivalence of the multiple-temperature and two-temperature methodologies in the determination of reaction volumes (Losi and Viappiani, 1998), the values we determined with the multiple-temperature method are systematically lower than the values obtained with the two-temperature method. For PLG the differences fall within the estimated uncertainties. However, in the case of the reaction volumes for neutralization of glu and acetate, the discrepancy appears to be significant, even after correction for the low concentrations of these acceptors used in multiple-temperature experiments (55 μM and 45 μM, respectively; and see Fig. 2). We have no good explanation for this observation. Although some evidence has been

TABLE 2 Structural volume and enthalpy changes for the deprotonation of *o*-nitrobenzaldehyde, ($\Delta V_{R,1}$, Q_1), and the neutralization of acetate, glu, and PLG (at different pH values and concentrations)

Proton acceptor	$\Delta V_{R,1}$ (ml/mol)	Q_1 (kcal/mol)	$\Delta V_{R,2}$ (mP/mol)	Q_2 (kcal/mol)	E_a (kcal/mol)	Frequency factor $\ln(k_2^0)$
Acetate	-7.4 ± 0.2*	95 ± 1	5.8 ± 0.1	2.0 ± 0.5	3.7 ± 0.5	21.6 ± 0.8
Glu	-6.7 ± 0.1	95 ± 1	4.5 ± 0.1	0.7 ± 0.4	4.9 ± 0.2	24 ± 1
PLG (pH 7.2)†	-7.0 ± 0.2	99 ± 1	11.6 ± 0.2	-3 ± 1	1.2 ± 0.4	23 ± 3
PLG (pH 4)†	-7.3 ± 0.2‡	96 ± 1‡	6.8 ± 0.1§	-1.4 ± 0.8§	1.6 ± 0.3‡	26 ± 2‡
					0§	15.9 ± 0.1§

$\Delta V_{R,2}$ and Q_2 were determined with the multiple-temperature method. The activation energy (E_a) and the frequency factor for each of the rates are also reported. The acetate and glu concentrations used were 45 and 55 μM, respectively.

*Uncertainty values were estimated as the standard deviations for three separate determinations of the parameter.

†These results are based on polymer concentrations.

‡Data from experiments at [PLG] = 2 μM, where cross-correlation between fitting parameters is negligible.

§Data from experiments at [PLG] = 40 μM, where the residual unimolecular reaction is better characterized (see text).

presented for a small temperature dependence of reaction volume (Millero, 1972; VanEldick et al., 1989), the excellent linearity of the plots used to derive the data in Table 2 (illustrated in Fig. 4) argues against such an explanation.

Kinetic results

The rate constants, k_b, for the neutralization reactions with glu and acetate are identical. For these compounds the rate k_b represents the diffusion controlled rate of the forward direction in reaction (5). The value of k_b for PLG listed in Table 1 is based on polymer molecule concentration units; when corrected for the average number of residues in the polypeptide ($n = 82$), the apparent k_b per residue was 3.4 ($\pm$ 0.1) $\times$ $10^{10}M^{-1}s^{-1}$. This simple estimate assumes that all of the residues are ionized and therefore will be most meaningful for the pH 7.4 result where the value obtained is smaller by almost a factor of two than the rate measured for the protonation of the simple carboxylates glu and acetate. This difference probably derives in part from the linkage of acceptors into large groups; thus, the protons must diffuse considerably farther on average before they encounter an acceptor (as compared to simple acceptors like acetate distributed uniformly throughout the solution). Values of k_b for proteins are also typically low. Examples are the rates for protonation of the carboxylates of BSA and RNase, 2.5×10^{10} $M^{-1}s^{-1}$, and for the C terminal and γ carboxylates of glu 35 in lysozyme, 1.2×10^{10} $M^{-1}s^{-1}$ (Gutman and Nachliel, 1990). Such low values probably result in part from shielding of the carboxyl groups by the globular protein structure.

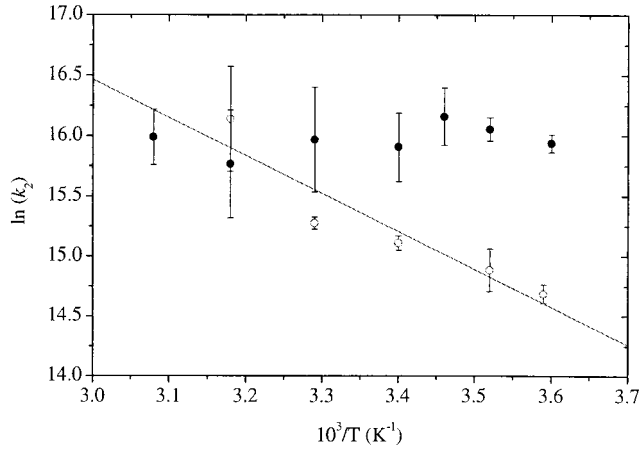


FIGURE 5 Arrhenius plots for the rate, k_b, associated with the binding of protons to PLG at pH 4 at [PLG] = 2 μM (*open circles*) and with the limiting rate, k_p, measured at pH 4 with [PLG] = 40 μM (*filled circles*). The activation energies calculated from the slopes are reported in Table 2. The results at high PLG concentration (*filled circles*) indicate that the activation barrier for the limiting rate is negligible in the temperature range investigated.

At neutral pH, the activation energies for proton binding to acetate and glu are very similar to one another but 3 to 4 times larger than the corresponding activation energy for PLG. This is to be expected considering the high negative charge density on PLG under these conditions.

Local helix formation in PLG

A substantially different process occurs when the reaction of photo-induced protons with PLG is investigated at prepulse pH around and below the pK_a of the helix-coil transition. For slight perturbations within the transition region of highly cooperative helix-coil conversions, the resulting relaxation process is determined by the growth reaction of already existing helix structures (Gruenewald et al., 1979). Binding of protons under these conditions can easily perturb the coil structure by removing electrostatically unfavorable interactions between charged residues and inducing the local formation of secondary structure around the binding site.

If proton binding to acceptors were solely responsible for the volume changes, the rate constant for protonation should increase linearly with PLG concentration until, above 30 μM, the volume changes due to proton binding would be unresolvable from the prompt events (as observed for glu

and acetate). However, when the PLG concentration is higher than 30 μM, the rate constant of the expansion reaches a plateau. Since the binding process under these conditions will be very fast (and therefore incorporated into the prompt response), this observation suggests that there is a unimolecular, rate-limiting step following proton binding.

The sign of the volume change is compatible with formation of local secondary structure as, for instance, a hydrogen bond between residues of the polypeptide. In fact, expansions are expected when secondary structure is formed and water is extruded from the interior of the more organized structure (Chalikian, 1996; Gross and Jaenicke, 1994; Chalikian et al., 1995; Kharakoz, 1989). The formation of hydrogen bonds is enhanced by reduction of the repulsive electrostatic interaction between charged carboxylates. The extent of the expansion associated with this local structure formation is 7.2 $\pm$ 0.9 ml/mol from two-temperature experiments and 6.8 $\pm$ 0.1 ml/mol from multiple-temperature experiments. It is unlikely that the structural volume change is simply reporting the formation of hydrogen bonds, for which a contraction of about -1 ml/mol has been reported (Gross and Jaenicke, 1994).

From the multiple-temperature experiments at pH 4 and high PLG concentration, we examined the temperature response of the rate constant for this rate limiting step. In the temperature range investigated, very low activation energy was indicated. In the Arrhenius plots reported in Fig. 5, it is striking to note the contrast between the temperature-dependent rate constants for the binding process ([PLG] = 2 μM) and the temperature-independent rate constant for the rate limiting step ([PLG = 40 μM). Evidently, the formation of local ordered secondary structure within already partially ordered sequences does not involve a significant energy barrier. In contrast, the activation energy for the helix-coil transition in poly-N^5-(3-hydroxypropyl)-L-glutamine (a neutral polypeptide) was measured as 16.7 kcal/mol (Gruenewald et al., 1979). Secondary structure formation requiring a nucleation processes has also been found to be thermally activated (Thompson et al., 1997).

We have recently reported for the acid-induced local disruption of the helical structure of poly-L-lysine a contraction of -16.8 ml/mol with a decay time of 250 $\pm$ 50 ns (limiting rate constant 4.0 ($\pm$ 0.8) $\times 10^6 s^{-1}$; Viappiani et al., 1998). The extent and sign of the volume change as well as the limiting rate constant observed for poly-L-lysine are fully compatible with the opposite structural change evidenced for PLG. The limiting rate constant for PLG is a factor of two larger than that measured for poly-L-lysine.

The lifetime at the midpoint of the helix-to-coil transition in PLG has been determined previously by means of ultrasonic absorption (1 μs), temperature jump (3 μs), and electric field jump (1.4 μs; Gruenewald et al., 1979). However, the local scale of the phenomena we have access to with our experiment is different from the global structural change monitored by these techniques, and a direct comparison is not easily justified. It is likely that the lifetime we have measured is representative of a faster process involving a local structural rearrangement in the region of the newly neutralized residue, constituting a limiting step in the helix growth reaction.

Recent data obtained for the rate constant of helix formation in model polypeptides by laser T-jump techniques can be compared to the data we obtained by laser pH-jump. Using the fluorescence emission of the N-terminal probe, 4-(methylamino)benzoic acid, Thompson et al. (1997) characterized the kinetics of the helix-coil transition of a 21-residue alanine peptide and identified a double exponential relaxation. The faster relaxation has been interpreted as due to the unzipping (and zipping) of the helix ends in response to the temperature jump, whereas the slower phase was associated with the equilibration of helix-containing and non-helix-containing structures by passage over the nucleation free energy barrier. The slower decay of the average helix content is in agreement with the data reported by Williams et al. (1996), who monitored the helix melting by infrared transient absorption in the amide I region and found for this process an apparent lifetime of about 160 ns.

Few experiments on helix formation in proteins have been reported in the literature. The thermally induced formation of helix A of apomyoglobin has been studied by Ballew et al. by monitoring the fluorescence emission of trp-14 (Ballew et al., 1996a,b). The authors found for the protective phase in the folding pathway an apparent lifetime of about 250 ns. Again, the rate constants appear in line with our determinations.

Finally, we wish to stress the similarity of the observed phenomena to our recent findings for apomyoglobin (Abbruzzetti et al., 2000). In that case, we induced a partial acid denaturation of native apomyoglobin at pH = 7. The early rearrangements after the ultrafast pH jump evidenced a rate-limiting step with a lifetime of about 1 μs and were postulated to involve the protonation of two hydrogen-bonded hystidines. The longer lifetime observed for that process is probably related to the movement of portions of the macromolecule larger than those of the poly-L-lysine and PLG samples studied.

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REFERENCES

Abbruzzetti, S., E. Crema, L. Masino, A. Vecli, C. Viappiani, J. R. Small, L. J. Libertini, and E. W. Small. 2000. Fast events in protein folding. Structural volume changes accompanying the early events in the N→I transition of apomyoglobin induced by ultrafast pH jump. *Biophys. J.* 78:405–415.

Abbruzzetti, S., C. Viappiani, D. H. Murgida, R. Erra-Balsells, and G. M. Bilmes. 1999. Non toxic, water soluble photocalorimetric reference compounds for UV and visible excitation. *Chem. Phys. Lett.* 304: 167–172.

Ballew, R. M., J. Sabelko, and M. Gruebele. 1996a. Direct observation of fast protein folding: the initial collapse of apomyoglobin. *Proc. Natl. Acad. Sci. USA.* 93:5759–5764.

Ballew, R. M., J. Sabelko, and M. Gruebele. 1996b. Observation of distinct nanosecond and microsecond protein folding events. *Nat. Struct. Biol.* 3:923–926.

Bonetti, G., A. Vecli, and C. Viappiani. 1997. Reaction volume of water formation detected by time resolved photoacoustics: photoinduced proton transfer between o-nitrobenzaldehyde and hydroxyls in water. *Chem. Phys. Lett.* 269:268–273.

Borsarelli, C. D., and S. E. Braslavsky. 1998. Volume changes correlate with enthalpy changes during the photoinduced formation of the (MLCT)-M3 state of ruthenium(II) bipyridine cyano complexes in the presence of salts. A case of entropy-enthalpy compensation effect. *J. Phys. Chem. B.* 102:6231–6238.

Braslavsky, S. E., and G. E. Heibel. 1992. Time-resolved photothermal and photoacoustics methods applied to photoinduced processes in solution. *Chem. Rev.* 92:1381–1410.

Callis, J. B., W. W. Parson, and M. Gouterman. 1972. Fast changes of enthalpy and volume of flash excitation of *Chromatium* chromatophores. *Biochim. Biophys. Acta.* 267:348–362.

Chalikian, T. V. 1996. On volume changes accompanying conformational transitions of biopolymers. *Biopolymers.* 39:619–626.

Chalikian, T. V., V. S. Gindikin, and K. J. Breslauer. 1995. Volumetric characterization of the native, molten globule and unfolded states of cytochrome c at acidic pH. *J. Mol. Biol.* 250:291–306.

Cocco, M., Y. H. Kao, A. Phillips, and J. T. J. Lecomte. 1992. Structural comparison of apomyoglobin and metaquomyoglobin: ph titration of histidines by NMR spectroscopy. *Biochemistry.* 31:6481–6491.

Creighton, T. E. 1990. Protein folding. *Biochem. J.* 270:1–16.

Gensch, T., and S. E. Braslavsky. 1997. Volume changes related to triplet formation of water-soluble porphyrins. A laser-induced optoacoustic spectroscopy (LIOAS) study. *J. Phys. Chem.* 101:101–108.

George, M. V., and J. C. Scaiano. 1980. Photochemistry of o-nitrobenzaldehyde and related studies. *J. Phys. Chem.* 84:492–495.

Gross, M., and R. Jaenicke. 1994. Proteins under pressure. The influence of high pressure on structure, function and assembly of proteins and protein complexes. *Eur. J. Biochem.* 221:617–630.

Gruenewald, B., C. U. Nicola, A. Lustig, and G. Schwarz. 1979. kinetics of the helix-coil transition of a polypeptide with non-ionic side groups, derived from ultrasonic relaxation masurements. *Biophys. Chem.* 9:137–147.

Gutman, M., and E. Nachliel. 1990. The dynamic aspects of proton transfer processes. *Biochim. Biophys. Acta.* 1015:391–414.

Hughson, F. M., D. Barrick, and R. L. Baldwin. 1991. Probing the stability of a partly folded apomyoglobin intermediate by site-directed mutagenesis. *Biochemistry.* 30:4113–4118.

Kharakoz, D. P. 1989. Volumetric properties of proteins and their analogs in diluted water solutions; partial volumes of amino acids at 15–55°C. *Biophys. Chem.* 34:115–125.

Laidler, K. J. 1987. Chemical Kinetics. HarperCollins Publishers, Inc., New York.

Losi, A., and C. Viappiani. 1998. Reaction volume and rate constants for the excited state proton transfer in aqueous solutions of naphthols. *Chem. Phys. Lett.* 289:500–506.

Millero, F. J. 1972. The Partial Molal Volumes of Electrolytes in Aqueous Solutions. R. A. Horne, editor. Wiley-Interscience, New York. 519–595.

Murgida, D. H., R. E. Balsells, P. R. Crippa, and C. Viappiani. 1998. Type I photosensitized reactions of oxopurines. kinetics and thermodynamics of the reaction with triplet benzophenone by time resolved photoacoustics. *Chem. Phys. Lett.* 294:538–544.

Pelagatti, P., M. Carcelli, and C. Viappiani. 1998. Determination of the pKa of the aci-nitro intermediate in o-nitrobenzyl systems. *Isr. J. Chem.* 38:213–221.

Peters, K. S., and G. J. Snyder. 1988. Time-resolved photoacoustic calorimetry: probing the energetics and dynamics of fast chemical and biochemical reactions. *Science.* 241:1053–1057.

Rasper, J., and W. Kauzmann. 1962a. Volume changes in protein reactions. I. Ionization reactions of proteins. *J. Am. Chem. Soc.* 84:1771–1777.

Rasper, J., and W. Kauzmann. 1962b. Volume changes in protein reactions. II. Comparison of ionization reactions in proteins and small molecules. *J. Am. Chem. Soc.* 84:1777–1788.

Rudzki, J. E., J. L. Goodman, and K. S. Peters. 1985. Simultaneous determination of photoreaction dynamics and energetics using pulsed, time-resolved photoacoustic calorimetry. *J. Am. Chem. Soc.* 107: 7849–7854.

Small, J. R. 1992. Deconvolution analysis for pulsed-laser photoacoustics. *In* Numerical computer methods. L. Brand and M. L. Johnson, editors. Academic Press, Inc., San Diego. 505–521.

Small, J. R., L. J. Libertini, and E. W. Small. 1992. Analysis of photoacoustic waveforms using the nonlinear least squares method. *Biophys. Chem.* 42:24–48.

Thompson, P. A., W. A. Eaton, and J. Hofrichter. 1997. Laser temperature jump study of the helix-coil kinetics of an alanine peptide interpreted with a 'kinetic zipper' model. *Biochemistry.* 36:9200–9210.

Van Eldick, R., T. Asano, and W. J. LeNoble. 1989. Activation and reaction volumes in solution. 2. *Chem. Rev.* 89:549–688.

Viappiani, C., S. Abbruzzetti, J. R. Small, L. J. Libertini, and E. W. Small. 1998. An experimental methodology for measuring volume changes in proton transfer reactions in aqueous solutions. *Biophys. Chem.* 73: 13–22.

Weast, R. C. 1968. CRC Handbook of Biochemistry. CRC Press, Boca Raton.

Weast, R. C. 1971. CRC Handbook of Chemistry and Physics. CRC Press, Boca Raton.

Williams, S., T. P. Causgrove, R. Gilmanshin, K. S. Fang, R. H. Callender, W. H. Woodruff, and R. B. Dyer. 1996. Fast events in protein folding: helix melting and formation in a small peptide. *Biochemistry.* 35: 691–697.