

Supplementary material to

Acid-induced unfolding of myoglobin triggered by a laser pH jump method

Stefania Abbruzzetti,^(a,b) Silvia Sottini,^(a,b) Cristiano Viappiani^(a,b) and John E.T. Corrie^(c)

(a) Dipartimento di Fisica, Università degli Studi di Parma, Parco Area delle Scienze 7/A,
43100 Parma, Italy

(b) CNR-INFN Parco Area delle Scienze 7/A, 43100 Parma, Italy

(c) National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

Photolysis of 2-NBA in the presence of BG

Figure 1 shows the absorbance changes at 633 nm of a solution containing 2-NBA, and BG at several laser pulse energies. The reaction occurs on sub- μ s time scale at all investigated laser pulse energies and its apparent rate increases with the laser pulse energy. It is clear, even at the lowest laser pulse energy, that protonation of more than 50 % of BG^- occurs, indicating that the concentration of the photoreleased protons brings the pH well below the pK_a of the pH indicator. The apparent rate constant is determined by the concentration of photodetached protons, the concentration of the other reactants, essentially 2-nitrosobenzoate anion ($ArCO_2^-$) and BG^- , as well as by the rate constants for the protonation reactions. The oscillations in curve C at times longer than $\approx 1 \mu$ s are due to acoustic effects induced by the absorption of the intense laser pulse.

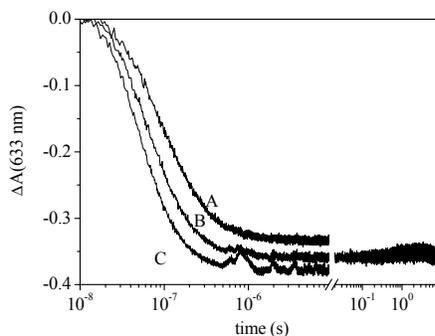
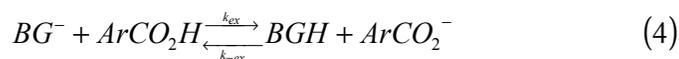
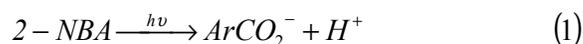


Figure 1. Transient absorbance at 633 nm following photolysis at 355 nm of an aqueous solution of 2-NBA and BG at pre-pulse pH = 6.9, T = 20 °C. Traces correspond to laser pulse energies of 12 mJ (A), 20 mJ (B), and 32 mJ (C). Trace B is shown with a wider temporal range to demonstrate the stability of the pH on longer time scale.

The time resolved absorbance traces reach stable values at approximately 1 μ s after photolysis of 2-NBA. This absorbance change remains stable for very long time scales, extending to several seconds, as demonstrated by trace B in Figure 1, which is shown up to $t = 10$ s. Under the present experimental conditions, mixing of the photolyzed volume with the remainder of the solution becomes effective for the ionic equilibrium of BG^- only at longer time scales. It is worthwhile pointing out that this is a substantial improvement with respect to the previous setup we used for studying the same reactions,^[1] for which mixing became evident on the time scale of 1 s. The major advantage of this very large dynamic range is the possibility of accessing a complete picture of the kinetics of the unfolding processes, which in many cases is extended in time.^[2, 3] For comparison, laser T-jump experiments are generally limited to the hundreds of milliseconds due to the tight focusing of the laser beams, which is necessary to achieve the desired temperature increase.^[4]

Data in Figure 1 can be used to retrieve the extent and the time course of the proton concentration change. Changes in absorbance (ΔA) of the pH indicator at 633 nm were converted to changes in concentration of the anionic form of the pH indicator ($\Delta[BG^-]$). Given that the deprotonation

reaction of 2-NBA occurs within a few nanoseconds, ^[5, 6] the proton transfer processes were modelled according to the following scheme:



where the reactions relative to the ionic equilibrium of water have been neglected given that our investigations dealt with pH values below neutrality, where the concentration of free hydroxide is low with respect to the other reactants. ^[11] In the above equations, $ArCO_2H$ and $ArCO_2^-$, represent the 2-nitrosobenzoic acid and its conjugate base. The pK_a for this acid has recently been determined ^[7] but the rate constant for either the protonation or the deprotonation reaction have not yet been determined experimentally. Both k_2 and pK_a were left as fitting parameters in our analysis. The formation of the nitronic acid ($pK_a = 2.1 \pm 0.2$ ^[5, 8]) occurs on sub-nanosecond time scale. ^[9-11] The deprotonation rate of this acidic intermediate is in the 10^8 s^{-1} range. ^[5] The change in concentration of free protons is therefore treated as instantaneous, and is accordingly introduced as an initial condition $[H_0^+]$. Formation of $ArCO_2^-$ is also considered instantaneous in the following discussion. ^[5, 12]

In order to estimate the dynamics and the extent of the proton concentration changes, we have analyzed simultaneously experimental protonation curves for BG induced by photolysis of 2-NBA taken at different laser pulse energies (from 10 mJ to 120 mJ). The numerical solution of the set of differential equations depends on the rate constants k_1 , k_2 , and k_{ex} , on the pK_a of the nitrosobenzoic acid, and on the concentration of the photodetached protons, $[H_0^+]$, proportional to the laser pulse energy. The rate constants k_1 , k_2 , and k_{ex} , and pK_a are common to all traces, whereas the values of $[H_0^+]$ are left free to vary from trace to trace. Rate constants k_{-1} , k_{-2} , and k_{-ex} have been introduced in the differential equations using the relationships $k_{-1} = k_1 10^{-pK_{a,BG}}$, $k_{-2} = k_2 10^{-pK_a}$, and $k_{-ex} = k_{ex} 10^{pK_a - 4.7}$.

Figure 2 shows the fitting of the experimental change in $[BG^-]$, after photolysis of *2-NBA*, to the solutions of the differential equations corresponding to reactions (1)-(4), at two selected laser pulse energies. The agreement between experimental data (circles) and fitted curves (solid lines) is excellent.

From the global analysis of the data for *2-NBA*, we have estimated the value of the rate constants k_1 , k_2 , and k_{ex} , pK_a , and the initial concentration of free protons $[H_0^+]$ as a function of laser pulse energy. The values we have obtained are $k_1 = (4.8 \pm 0.5) \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$, $k_2 = (1.9 \pm 0.2) \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$, and $k_{ex} = (4.5 \pm 0.4) \times 10^9 \text{ M}^{-1}\text{s}^{-1}$. The rate constant k_1 is in agreement with our previous determination $(5.9 \times 10^{10} \text{ M}^{-1}\text{s}^{-1})$ [1] and with other literature data $(4.2 \times 10^{10} \text{ M}^{-1}\text{s}^{-1})$ [13]. The value of k_{ex} is lower than k_1 and is comparable with the values reported for similar proton exchange reactions occurring between a charged and a neutral species. [1, 13] The value of k_2 is also in line with what is expected for a carboxylic acid. [13-15] In Figure 2 we have reported also the time profile of the changes in concentration of $ArCO_2^-$, and the change in concentration of free protons from its initial value $[H_0^+]$. The change in free proton concentration is mostly due to the protonation of $ArCO_2^-$, which effectively reduces the concentration of protons available for further reactions.

From the global analysis we could also estimate the pK_a of the nitrosobenzoic acid, $pK_a = 3.8 \pm 0.3$, a value which is in good agreement with the recent determination of 3.64 by Pytela and Kulhanek. [7]

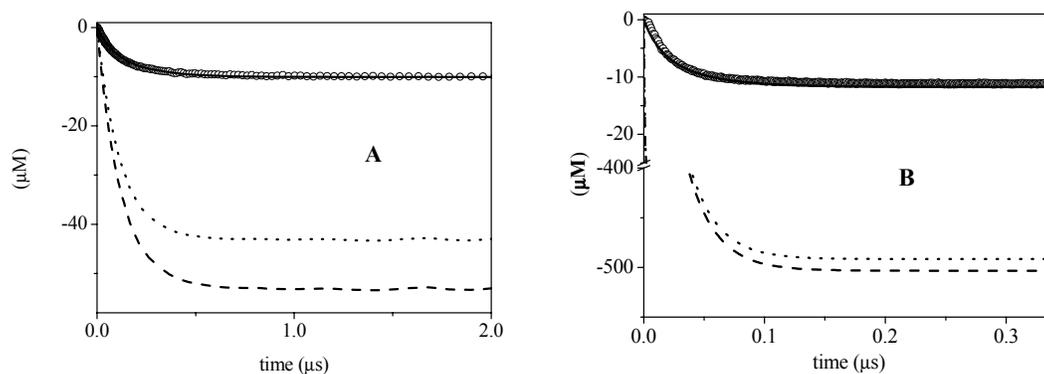


Figure 2. Fits of the time course of $\Delta[BG^-]$ in μM units after the laser pulse, for traces taken at two different energies (A: 12 mJ; B: 120 mJ). Open circles are the experimental data, solid lines are the fits to the concentration changes, dotted lines are the time courses of $\Delta[ArCO_2^-]$ and dashed lines are the time courses of the proton concentration change $\Delta[H^+]$. Pre-pulse pH = 6.9, $T = 20\text{ }^\circ\text{C}$.

Figure 3 evidences this scavenging effect by comparing the values of $[H_0^+]$ and the concentration of free protons at $t = 1\ \mu\text{s}$ (i.e after the equilibrium has been reached) as a function of laser pulse energy. The concentration of photoreleased protons can reach the value of $\approx 0.86\ \text{mM}$ at the pulse energy of 120 mJ. Out of these protons, only $\approx 0.46\ \text{mM}$ survive free in solution $1\ \mu\text{s}$ after photoexcitation. It appears therefore evident that, although protons are released with high efficiency, buffering by the photoproduct is very effective and reduces their availability. So, if the pH of the solution is ≈ 3.1 at the end of the laser pulse, $1\ \mu\text{s}$ after photoexcitation the pH of the solution has equilibrated to ≈ 3.3 .

It should be also emphasized that the pH indicator itself is a buffer for photoreleased protons. However, due to its low concentration, scavenging of protons from the solution is not as effective as in the case of the photoproduct. Buffering effects are expected to be substantial in studies with complex polyelectrolytes as proteins, in which the concentration of protonatable sites easily reaches the $10^{-4}\ \text{M}$ range (*vide infra*).^[15-17]

The data shown in Figure 3 demonstrate that there is a steady increase in the concentration of photoreleased protons as a function of laser pulse energy. The increase is not linear in the high energy range, where saturation effects become evident. The molar absorption coefficient of *2-NBA* at 355 nm is very low ($\approx 500\ \text{M}^{-1}\text{cm}^{-1}$) and this allows to minimize saturation effects due to the concentration of the photolabile precursor.

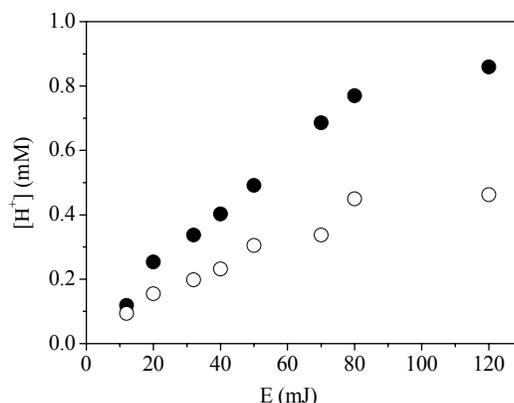


Figure 3. Changes in free proton concentration at the end of the laser pulse (filled circles) and at $t = 1 \mu\text{s}$ (open circles) as a function of laser pulses energy, following photolysis of 2-NBA. Pre-pulse $\text{pH} = 6.9$, $T = 20 \text{ }^\circ\text{C}$.

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