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PROTON TRANSFER TO A CHARGED DYE BOUND TO THE α-CHYMOTRYPSIN ACTIVE SITE STUDIED BY LASER PHOTOLYSIS

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Summary

Pulsed laser photolysis has been used to study the very rapid relaxation of the complex of α-chymotrypsin (EC 3.4.21.1) with the coloured inhibitor Biebrich Scarlet. The light absorption causes the dissociation of the proton in the dye naphthol ring and we are able to follow the recombination process under conditions of different ionic strength and pH. The recombination is markedly influenced by the pH around pH 7. The data suggest the existence of relevant interactions in the active site area between the hydrophobic binding site and the proton relay system of the enzyme.

Introduction

Photoinduced reactions in enzyme-inhibitor complexes have recently been successfully used to study specific interactions at the active site of enzymes [1,2]. A coloured substrate analogue, bound to the active site, could provide a useful and sensitive way to initiate a local specific reaction after the absorption of one photon in the visible region.

This method has a quite general applicability in the study of rapid molecular processes occurring in enzymatic catalysis. A relevant class of such processes are proton transfer reactions, which are often too fast to be studied by classical methods. Pulsed laser methods allow a selective initiation of the reaction and the possibility to detect very rapid relaxation effects [3—5].

In this work we study the α-chymotrypsin-Biebrich Scarlet complex with a pulsed laser photolysis apparatus, already described [6], which has a resolution time of less than 100 ns. The absorption of one photon, corresponding to the first $\pi \rightarrow \pi^*$ singlet transition in the dye, causes the dissociation of the proton in the naphthol ring. Following the recombination rate in different ionic strength and pH conditions we are able to get informations about the processes occurring in the dye microenvironment.

Materials and Methods

Crystalline salt-free enzyme, obtained from Boehringer, was used for preparing solutions of α-chymotrypsin. Most of the experiments were carried out at enzyme concentrations below 10^{-4} M. At these values the amount of enzyme autolysis, observed as a decrease in the signal amplitude or in the relaxation time, was negligible after some days. However, the experimental measurements were made immediately after the preparation of the samples.

The organic dye Biebrich Scarlet was obtained from BDH Chemicals Ltd.

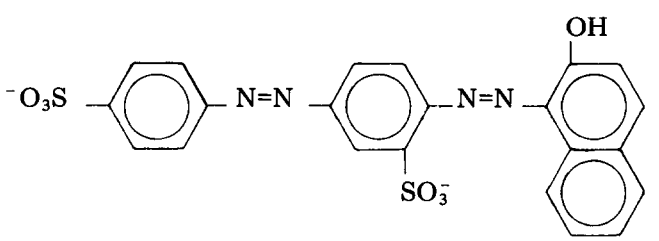
The absorption spectra were recorded on a DBGT Spectrophotometer provided with a thermostatically temperature-regulated cell holder.

The sample solutions had the following composition: α-chymotrypsin $4.5 \cdot 10^{-5}$ M, Biebrich Scarlet $4.5 \cdot 10^{-5}$ M, buffer P_i at different pH and concentration. Proton transfer effects were studied in quite highly buffered media (0.01—0.1 M phosphate). In this conditions considerable ionic strength variations occur in pH titration curves. The effect of this variation however was confined inside instrumental errors as is seen by addition of 0.1 M NaCl to a sample with 0.01 M phosphate buffer at pH 7.

The pulsed laser has been described in detail elsewhere [6]. In this work we used the λ = 532 nm output wavelength obtained from the fundamental Nd 1.06 μ line by frequency doubling. The pulse width produced by the Q-switch was 80 ns, with a maximum peak power of 1.5 kW corresponding to an energy of 0.1 mJ per pulse; the repetition rate was 70 Hz. The sample cell was 1 mm thick and placed at Brewster angle with the laser beam. The process was monitored in the 335—460 nm absorption bands of the complex with a high sensitivity spectrophotometric detection system [6]. The signal was analysed electronically by a sampling method and averaged on thousands of laser pulses to enhance the signal/noise ratio, from values around unity to ten or so. The observed photochemical process is near the equilibrium, and consequently the observed relaxations are always exponential. The relaxation time constants are obtained from the slope of semilogarithmic plots of the signal and directly evaluated by computer optimization.

Results and Discussion

Biebrich Scarlet has been proved to be a specific inhibitor of α-chymotrypsin [7]. The dissociation constant, $K_d = 8.8 \cdot 10^{-5}$ M, allows preparation of samples where most of the dye is bound to the enzyme. It is suitable for photolysis experiments because of its strong absorption in the 500 nm region. The structural formula is the following:



In free aqueous solutions the proton dissociation constant of the naphthol ring is quite low ($pK > 11$) compared to the normal values in naphthol molecules ($pK \approx 9.5$ [8]) since the presence of the negative charge of the sulphonic groups enhances the affinity for the proton.

The photochemical effect resulting from the laser light pulse absorption in the α-chymotrypsin-Biebrich Scarlet complex (shown in Fig. 1) consists in an abrupt decrease in the 430—500 nm absorption bands, and in an increase in the 330—360 nm bands, followed by a single exponential relaxation with a time constant of some μs depending on the pH and the ionic strength. No other relaxations have been detected in the temporal range covered by the apparatus (0.1—100 μs).

The effect is probably caused by a process occurring in the enzyme-dye complex, since: (1) It is not present in the dye alone. In $4.5 \cdot 10^{-4}$ M solutions of free dye in phosphate buffer pH 8, only a very small photochemical effect could be detected with an amplitude almost 20 times smaller than the one observed with chymotrypsin-dye complex and with a very short relaxation time constant ($\tau \approx 600$ ns). (2) The amplitude of the effect shown in Fig. 1 varies linearly with the enzyme-inhibitor concentration. (3) The rate constant is too great to be a binding process of the dye to the enzyme. Even assuming a diffusion-controlled rate constant of 10^9 $s^{-1} \cdot M^{-1}$ the binding rate constant would be $3 \cdot 10^4$ s^{-1} at our dye concentration which is considerably smaller than the observed value. Nor can the self-aggregation of the dye [9] contribute since no effect is detected with the dye alone. Furthermore, all processes involving the free dye are kinetically uncoupled to the observed effect because of the low rate constant of the binding processes.

The amplitude of the photochemical process as a function of the observation wavelength is reported in Fig. 2, together with the difference spectrum of the

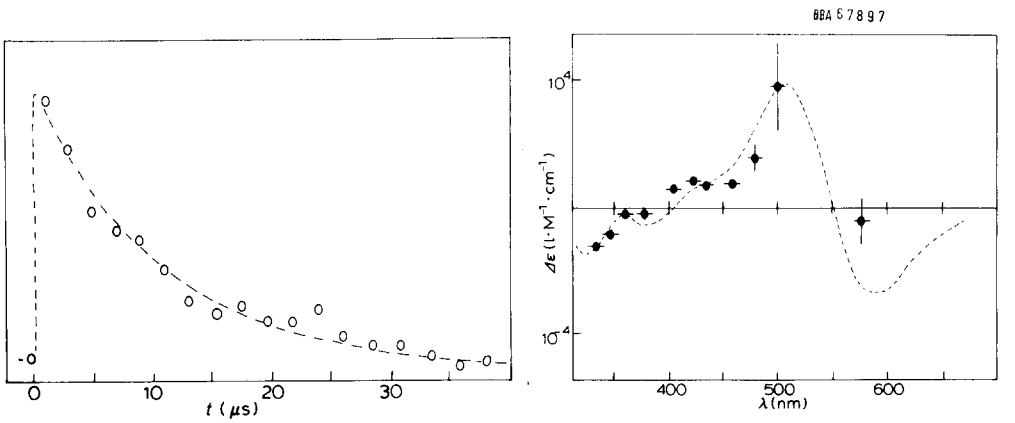


Fig. 1. Photodissociation effect on the α-chymotrypsin · Biebrich Scarlet complex. Laser wavelength λ = 532 nm, observation wavelength λ = 435 nm. Enzyme and dye concentration: $4.5 \cdot 10^{-5}$ M, phosphate buffer; 9 mM, pH = 7.6.

Fig. 2. Amplitude of the photochemical effect vs. observation wavelength. The curve (- - - - -) is the differential spectrum between pH = 7 and pH = 12 of the free dye in aqueous solution.

free dye at pH 7 minus the free dye at pH 12.2. These data indicate that the photochemical effect is caused by a laser-induced photodissociation of the naphthol proton. The process is described by a Förster cycle [8] with a large pK shift between the ground and the excited state. This is a general characteristic of naphthol derivates [8]. The quantum yield of the photoreaction can be roughly estimated, assuming that the variation of the molar extinction coefficient due to the dye ionization is the same in the free and in the bounded form. The resulting value is about 0.01.

To explain the absence of the signal with the dye alone we postulate the existence of a strong shielding effect on the sulphonic group of some positively charged residue at the enzyme active site [10]. Since the latter neutralizes the negative charge of the sulphonic group, the affinity of the naphthol ring for protons is much lower in the enzyme-bound than in the free dye, and the rate constant for proton dissociation both in the ground and in the excited states are correspondingly enhanced.

We do not know the molecular details of the dye interaction with the active site of the enzyme; however we can reasonably interpret the equilibrium spectrophotometric studies [7] in terms of the known X-ray structure [11]. In this case, if the dye is bound to the enzyme similarly to the analogous inhibitors shown in the X-ray structures [10—12], side-chain of Ser 195 could be near to the sulfonic group; this was suggested also by the fact that modification of this serine residue prevents the dye binding to the enzyme [7]. On the other hand the naphthol ring will be probably be bound inside the hydrophobic pocket as is suggested not only by analogous complexes with inhibitors observed by X-ray diffraction [11,12], but also by the fact that Biebrich Scarlet competes with indole [7], and does not bind to the chymotrypsinogen molecule [7,13].

The behaviour of the reciprocal relaxation time constant for the recombination process of protons to the dye naphthol ring is shown in Fig. 3 as a function of ionic strength at two different pH values. We note that: (1) the linear dependence with the ionic strength is characteristic of a buffer-mediated proton transfer described by the scheme:

$$E + BH \underset{k^+}{\overset{k^-}{\rightleftharpoons}} EH + B \qquad (1)$$

where E and EH are the unprotonated and protonated species of the enzyme-dye complex, B and BH the buffer anion and the corresponding base respectively; $k^- \gg k^+$ because of the quite high pK of the naphthol proton. (2) The time constant for the proton recombination, calculated according to eqn. 1, is

$$1/\tau \simeq k^- \cdot [BH].$$

From the data of Fig. 3, taking into account correction for the activity due to the ionic strength, we derive $k^- = 4.0 \pm 0.4 \cdot 10^6\ s^{-1} \cdot M^{-1}$ at pH = 7, which is more than two orders of magnitude slower than expected if the proton exchange between buffer molecules and enzyme were a diffusion-controlled reaction [14]. It is most likely that the naphthol ring of the dye is located in a position inside the hydrophobic pocket of the enzyme, so that the direct contact with the buffer molecules is hindered.

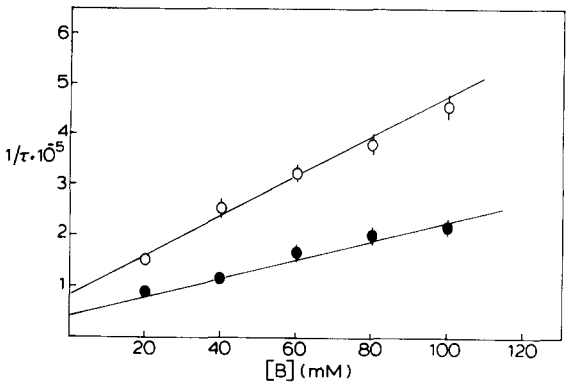


Fig. 3. Proton recombination rate constant vs. buffer concentration: (●) pH = 7, (○) pH = 8.

The linear dependence of the inverse relaxation time with buffer concentration could also of course be described assuming schemes more complicated than eqn. 1. A more realistic and complete picture of the enzyme interactions with the ionic medium should include eventual intramolecular proton transfer and/or anion-binding processes. However, the increases of the ionic strength by salt addition do not significantly influence the observed rate constant (see Materials and Methods). The observed buffer dependence requires then proton transfer between dye and buffer molecules, and this effect could be described by some overall phenomenological constant k^- in any case.

The influence of the pH on the rate constant of the process at two different buffer concentrations is described in Fig. 4. The observed dependence upon pH is mainly due to a change in the k^- rate constant of eqn. 1. This behaviour shows that the proton uptake at the naphthol ring of the dye is influenced by the protonation of some group with a pK of approx. 7.5. The sigmoidal shape is particularly steep at high ionic strength. This effect could be due either to cooperative ionization of more than one charged group on the protein, and/or to counterion binding. Our data cannot discriminate between these two contributions because of instrumental imprecision.

The existence of a ionizable group near the dye-binding region is also confirmed by the absorption spectra of the dye · enzyme complex which show an appreciable variation with pH. This group is probably His 57 which is known to be essential in the catalytic mechanism [15—17]. We are led to assess the existence of an interaction among the enzyme groups at the active site, and in particular between the hydrophobic pocket, where most probably the naphthol ring is bound, and the proton relay system [15]. This interaction could be of an electrostatic and/or conformational nature [18,19]. However in the molecular picture that we get from our measurements the zone of interaction of the residues of the active site center extends well beyond the Asp 102—His 57—Ser 197 region. Furthermore the presented data are a direct evidence of the essential role of the interactions between charged residues of the protein and the ionic medium.

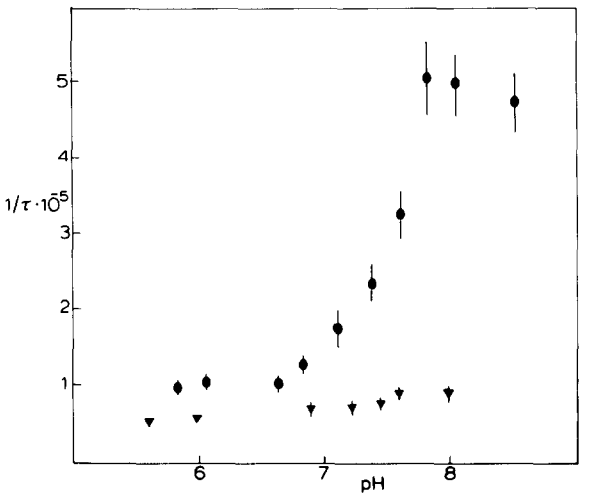


Fig. 4. pH dependence of the proton recombination rate constant at two different buffer concentrations, (●) phosphate buffer 90 mM, (▲) phosphate buffer 9 mM.

Acknowledgements

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