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Investigation of Lysozyme-Chitobioside Interactions Using Synchronous Luminescence and Lifetime Measurements

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Beside their ability to disrupt the outer membranes of some microorganisms, lysozymes also experience interactions with chitins or their fluorescent analogs. It has been well established that chitins bind to the cleft of lysozymes and the subsites of the location of the different *N*-acetylglucosamines that are parts of chitins have been identified. Moreover, it has been well documented that a 1,4-β-bond must be located between subsite D and subsite E to be cleaved. Nevertheless, a better understanding of the biophysical and biochemical processes is needed.

In this paper, pulsed fluorescence was used to further investigate the mechanism by which the binding of fluorescent analogs of chitin (4-methylumbelliferyl chitobiose and 4-methylumbelliferyl chitotriose) to hen egg-white lysozyme results in an increase of their fluorescence intensity. Although such an increase is not observed when these chitobiosides bind to turkey egg-white lysozyme, synchronous fluorescence techniques show that this binding induces a quenching of the native fluorescence of both these proteins.

The findings of this study, associated with previously published cyrstallographic data allow us to suggest that the system lysozyme-chitobioside partitions in two three-dimensional conformational states: an enzymatic active conformation and a storage conformation. These states are separated by an energy barrier, with the storage conformation being more populated than the enzymatic active conformation below 45°C.

KEY WORDS: Hen and turkey egg-white lysozyme; chitobiosides; 3D conformational state; pulsed fluorescence; synchronous fluorescence.

INTRODUCTION

There have been numerous investigations on the lysozyme-catalysed reactions of various substrates [1–19]. However, complete understanding of lysozyme catalysis has not yet been achieved owing to the complexity and relative insensibility to lysozyme of the substrates used, as well as strong nonproductive binding, and the transglycosylation reaction [18–22] that occurs simultaneously with the hydrolytic reaction.

In addition to being 1,4-β-*N*-acetylmuramidases, lysozymes are also capable of hydrolyzing chitin, the 1,4-β–linked homopolymer of *N*-acetylglucosamine (GlcNAc). Fluorogenic substrates such as 4-methylumbelliferyl (4-MeU) β-glycosides of chitin have been increasingly used for detection of chitinase activity [23–25]. Only the hydrolytic reaction at the aryl glycosidic bond between the GlcNAc residue and the 4-MeU group is measured fluorimetrically, which makes these substrates easy to use in kinetic measurements.

Whereas avian lysozymes have been used in most of the previous studies, structural studies with rainbow

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trout lysozyme (RBTL) and a variety of oligosaccharides have also been carried out [26–29] to obtain more information about the catalytic mechanism. In each of these studies, only non-productive binding modes in sites A–D of the active-site cleft have been observed. The results from these studies question whether a distorted sugar conformation in site D is necessary for effective catalysis, as has been proposed by Blake *et al.* [30].

Although the crystal structure of the complexes between RBTL and 4-methylumbelliferyl chitobiose (4MeU(Glc)2) and 4-methylumbelliferyl chitotriose (4MeU(Glc)3) have been elucidated by Vollan *et al.* [29], the present study is devoted to the interaction of these compounds with hen egg-white lysozyme (Hlys) and turkey egg-white lysozyme (Tlys) respectively. In both cases the simplest kinetic scheme may be expressed as:

$$ES_{np} \overset{KES_{np}}{\rightleftarrows} E + S \overset{KES_{p}}{\rightleftarrows} ES_{p} \overset{k_2}{\rightarrow} E + P$$

where ES_{np}, and ES_p represent nonproductive ES and productive ES complexes, respectively, $K\,ES_{np}$ and KES_p represent the dissociation constants of the non-productive and productive complexes, respectively; and k_2 is the rate constant for the rate-limiting step. The dissociation constants for the interaction of hen egg-white lysozyme with 4MeU(Glc) and 4MeU(Glc)3, at 298 K and pH 5.18, were calculated by Yang and Hamaguchi [25], by taking advantage of the increase of the intensity of fluorescence of these substrates when bound to the protein. The dissociation constants were determined to be 66.7 μM and 20 μM for 4MeU(Glc)2 and 4MeU(Glc)3 systems, respectively.

Although the amino-acid sequence of Hlys and Tlys are very similar, neither 4MeU(Glc)2 nor 4MeU(Glc)3 exhibited any increase in fluorescence intensity upon binding with Tlys. As a result, Yang and Hamaguchi were unable to get any information about the thermodynamics of these interactions.

In this paper, interactions of 4MeU(Glc)2 and 4MeU(Glc)3 with hen egg-white lysozyme and turkey egg-white lysozyme are studied using lifetime measurements and synchronous fluorescence techniques. Lifetime measurements suggest that the increase in fluorescence intensity results from a decrease in the vibrational deactivation process when the fluorescent substrates are bound to Hlys. Beside the previously mentioned increase in the fluorescence intensity experienced by both fluorescence substrates when bound to Hlys, a strong decrease in the fluorescence intensity of the protein is observed. This decrease reveals a quenching of the fluorescence of some tryptophan residue(s). This quenching is not associated to an energy transfer from these excited tryptophan(s) to the fluorescent substrate. The completion of the quenching occurs for a ratio of the substrate/protein concentration lower than that necessary to saturate the increase of the fluorescence intensity of the substrate, suggesting that these effects signify different molecular events. Moreover, such a quenching is also observed when both fluorescent substrates bind to Tlys, although this interaction does not induce any measurable change in the fluorescence intensity of the substrates. Results are discussed in terms of an equilibrium between two three-dimensional conformations of these lysozymes.

MATERIALS AND METHODS

Chemicals

The 4-methylumbelliferyl β-D-*N,N′*diacetylchitobioside (4MeU(Glc)2), 4-methylumbelliferyl β-D-*N,N′N″*diacetylchitotrioside (4MeU(Glc)3) hen egg-white lysozyme, (Hlys), and turkey egg-white lysozyme, (Tlys) were obtained from Sigma (Saint Quentin Fallavier, France). Stock solutions (347 μM) of 4MeU(Glc)2, (264 μM) of 4MeU(Glc)3, were prepared by adding 5.00 ml of water to 1.00 mg of the probe. All the stock solutions were kept between 0°C and 4°C.

Excitation, Emission and Synchronous Fluorescence Spectroscopy

Excitation and fluorescence spectra were digitally recorded with either a Jobin-Yvon JY3D spectrofluorometer, interfaced with a Tandon AT 286 microcomputer; or a LS 50 Perkin Elmer spectrofluorometer. Instead of being measured from the peak height for each spectrum, the fluorescence intensities reported in the figures were determined from the area beneath a fluorescence band, to improve the sensitivity and data accuracy.

Synchronous fluorescence spectra for proteins with and without fluorescent substrates were recorded with a $\delta\lambda = 40$ nm [31]. Figure 1 shows that this accurate and fast method allows a good separation between the respective spectrum of the protein and that of its substrate.

Fluorescence Lifetime Measurements

A pulsed nitrogen laser (LSI VSL 337 WD) coupled to a fast photomultiplier (RCA 1P28) monitored with an oscilloscope (Tecktronix TMS 350) was used for these measurements. Numerical signals were then analyzed using a program based on modulating functions, pre-

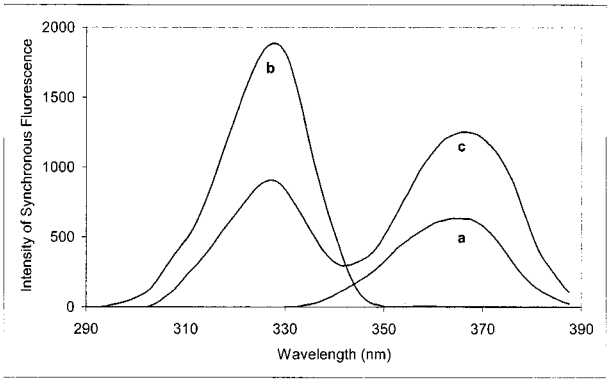

Fig. 1. Respective synchronous fluorescence spectra ($\delta\lambda$ = 40 nm) of a solution of 4MeU(Glc)2 (1.05 μM) (curve **a**), a solution of Hlys (15 μM) (curve **b**), and a solution of Hlys (15 μM) in the presence of 4MeU(Glc)2 (1.05 μM) (curve **c**). Using the synchronous fluorescence technique allows a good separation of the native fluorescence spectrum of Hlys that peaks at 325 nm from that of 4MeU(Glc)2 free or Hlys-bound that peaks at 367 nm. The presence of 4MeU(Glc)2 results in a decrease of the fluorescence of Hlys, whereas the presence of a large excess of Hlys results in an increase in the fluorescence of 4MeU(Glc)2.

viously developed in one of our laboratories (University of Perpignan).

RESULTS AND DISCUSSION

1°-Fluorescence Studies of the Chitobioside in the Presence of Hen Egg-white Lysozyme

Yang and Hamaguchi have used the increase of the fluorescence intensity of the chitobiosides and chitotriosides to calculate the association constant, Ka, of the interaction of these fluorescent substrates with hen lysozyme. Yet the cause of this increase was not reported. So in the first step of our study, the synchronous fluorescence spectrum of a solution of Hlys, 15 μM; pH, 5.4; temperature, 22°C, was compared with that of the same solution in the presence of 4MeU(Glc)2, 1.05 μM. As shown in Fig. 1, the binding of the fluorescent substrate to the enzyme causes a quenching of the enzyme fluorescence. It is worthy to note that, because in synchronous fluorescence techniques both excitation and emission wavelengths are varied at the same time, the method is insensitive to a potential energy transfer from the excited Hlys-belonging tryptophan(s) to the fluorescent moiety of the substrate (Fig. 2). Such an energy transfer was specifically searched for by recording the excitation spectrum of a more concentrated solution, (40 μM in Hlys and 8.0 μM in 4MeU[Glc]2). The emission wavelength was fixed at the maximum of the fluorescence spectrum of 4MeU(Glc)2 while the excitation wavelength was varied from 250–310 nm to cover the excitation domain of tryptophans. Furthermore, the excitation spectrum of a solution 8.0 μM in 4MeU(Glc)2 without Hlys was also recorded. Both spectra are shown in Fig. 3. As demonstrated by the figure, the sum of the respective excitation intensity of the solution with Hlys alone, and that of the solution with 4MeU(Glc)2 alone, is higher than the intensity of the solution where they are both present. This result illustrates the exclusion of any energy transfer between Hlys and the substrate. Therefore the increase of the fluorescence intensity of 4MeU(Glc)2 seems to result from a restriction of the vibrational deactivation of the excited state of the substrate because of the location of the sugars in the cleft that contains the active site of the enzyme (Fig. 2). This increase is not directly related to the quenching of the fluorescence of the enzyme that is also associated with the binding of that fluorescent substrate. Nevertheless, whether these two phenomena are referred to the same “binding site” remains an open question.

Lifetime measurements were also performed on solutions of 4MeU(Glc)2 and 4MeU(Glc)3 1.0 μM, alone and in the presence of a large excess of Hlys or Tlys (100 μM). They demonstrated that in the presence of Hlys the intensity of the fluorescent pulse of these chitobiosides is strongly enhanced. Data obtained for 4MeU(Glc)3 are displayed in Fig. 4A. On the contrary, the presence of Tlys did not induce any significant change. This finding is consistent with the fact that the presence of Tlys did not induce any change in the intensity of the fluorescence spectrum of any of these chitibiosides [25]. Furthermore, this enhancement was lower for pH 7.6 than for pH 5.4, suggesting that the ionization of the amino-acid residue Glu35 increases the degree of freedom of the dye (data not shown).

In the absence of lysozyme, the optical signal issued from the photomultiplier can be modeled as the convolution product of the pulse of excitation by a mono-exponential decay (τ = 0.6 ns) (data not shown). In the presence of a large excess of Hlys, the decay involves two exponential functions, one of them very short (τ = 0.8 ns) with a high relative weight, and the other in the range of 6.5 ns (Fig. 4B). This phenomenon is not associated with a change in shape and position of the fluorescence spectrum of the chitobiosides. So, such a finding confirms the idea that the increase in intensity observed by Yang and Hamaguchi results from a decrease in the efficacy of the non-radiative deactivation process that occurs mainly in the Frank-Condon state of the excited molecule. This decrease in efficacy of the non-radiative deactivation process could result from a

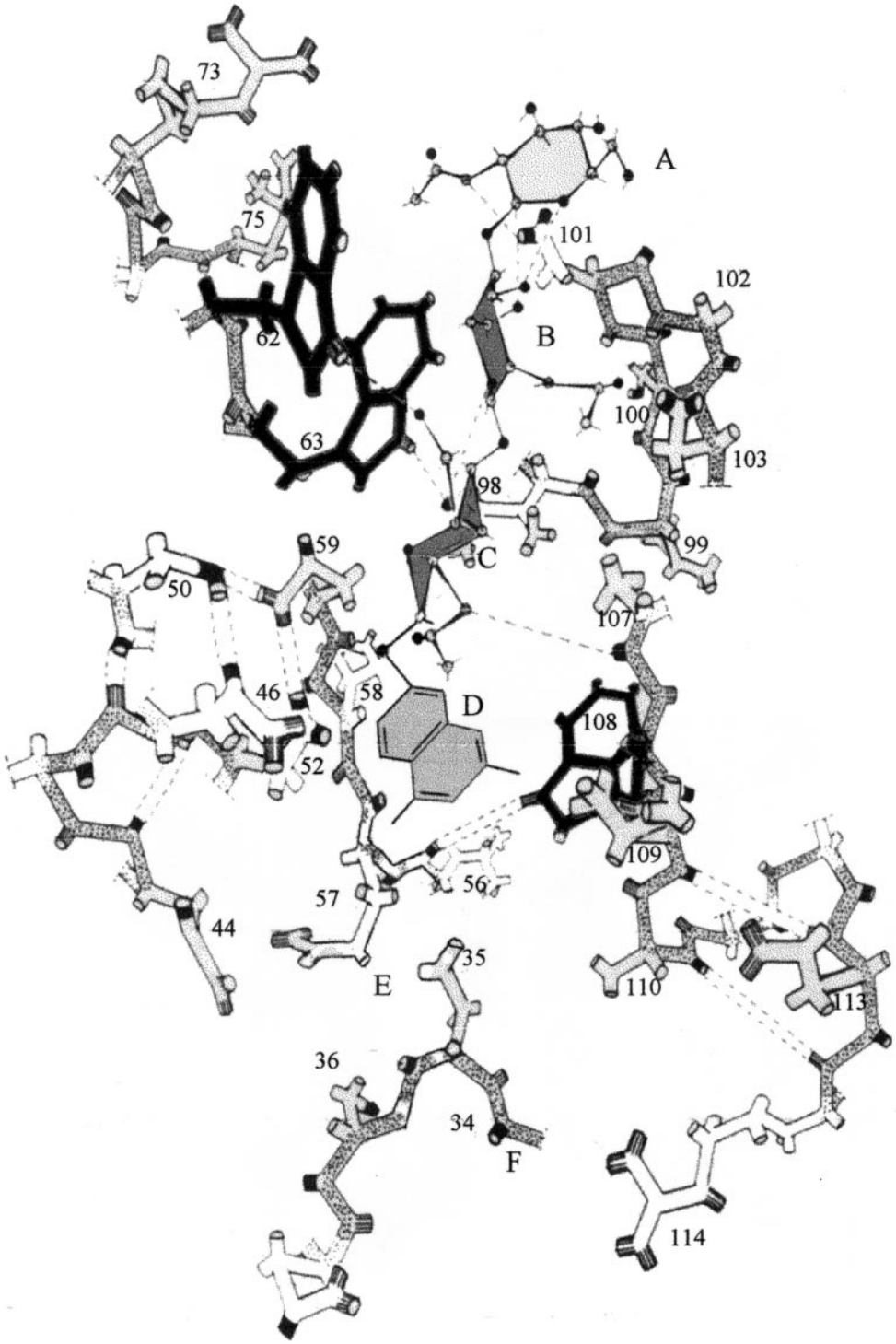

Fig. 2. Schematic drawing of the interaction between 4MeU(Glc)3 or 4MeU(Glc)2 and lysozymes, adapted from Phillips (1967) and Vollan *et al.* (1999). The fluorescent parts of the substrates are in green, the sugars belonging to both 4MeU(Glc)3 or 4MeU(Glc)2 are in orange, and the extra sugar of 4MeU(Glc)3 is in yellow. The tryptophan residues potentially involved in the quenching of the fluorescence of the protein are in blue. Letters in red refer to the location subsites of sugars.

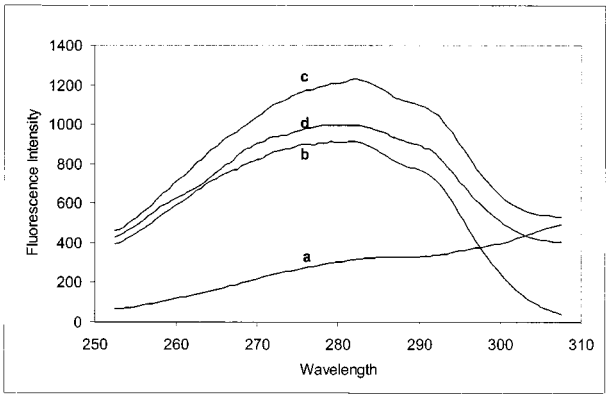

Fig. 3. Respective excitation spectra of a solution 8 μM in 4MeU(Glc)2 (curve **a**) and of a solution 40 μM in Hlys (curve **b**). The sum of these two curves (curve **c**) is higher than the excitation spectrum of a solution 40 μM in Hlys and 8 μM in 4MeU(Glc)2 (curve **d**), demonstrating that no energy transfer occurs from the tryptophans of the protein to the fluorescent substrate. (λem = 370 nm.)

decrease in the rotational-vibrational degree of freedom of the oligosaccharide chain because of its location inside the cleft of the protein (Fig. 2).

2°-Fluorescence Studies of Hlys (2 to 15μM) in the Presence of 4MeU(Glc)3 at Room Temperature (pH 5.4)

In a preliminary set of experiments the synchronous fluorescence spectrum of solutions with different concentrations of 4MeU(Glc)3 were recorded in the absence of any Hlys. Then a daily prepared stock solution 100 μM in Hlys was used to prepare a 15-μM solution of Hlys without 4MeU(Glc)3 and a solution 15 μM in Hlys and 25 μM in 4MeU(Glc)3. These solutions were used to prepare solutions of intermediate concentrations in 4MeU(Glc)3 and constant concentration of Hlys. The synchronous fluorescence spectra of each of these solutions were then recorded. As shown in Fig. 5, the fluorescence spectrum of the protein, 270–300 nm, decreases as the concentration in 4MeU(Glc)3 increases. On the contrary, a concomitant increase in the long wavelength range of the fluorescence spectrum ($\lambda > 300$ nm) is observed as a result of the presence of both free and protein-bound 4MeU(Glc)3. Comparison with the fluorescence intensity of solutions with the same concentration in 4MeU(Glc)3, but without Hlys, allows us to calculate the increase in fluorescence intensity of 4MeU(Glc)3 resulting from its binding to Hlys for each 4MeU(Glc)3 concentration. As expected, the Ka value (20 μM) proposed by Yang and Hamaguchi allows a proper modeling of this increase in fluorescence intensity (Fig. 6). On the contrary, the quenching of the protein fluorescence intensity cannot be modeled using only one association constant. It is necessary to introduce another binding constant ($Ka_2 = 0.4$ μM), which suggests two different conformations for the complex protein-substrate (Fig. 7). The agreement between the experimental and theoretical plots demonstrated that the quenching of the protein fluorescence and the increase of intensity of the substrate result from two different phenomena. Yang and Hamaguchi have suggested that the change in the fluorescence intensity of the chitobiosides may be correlated

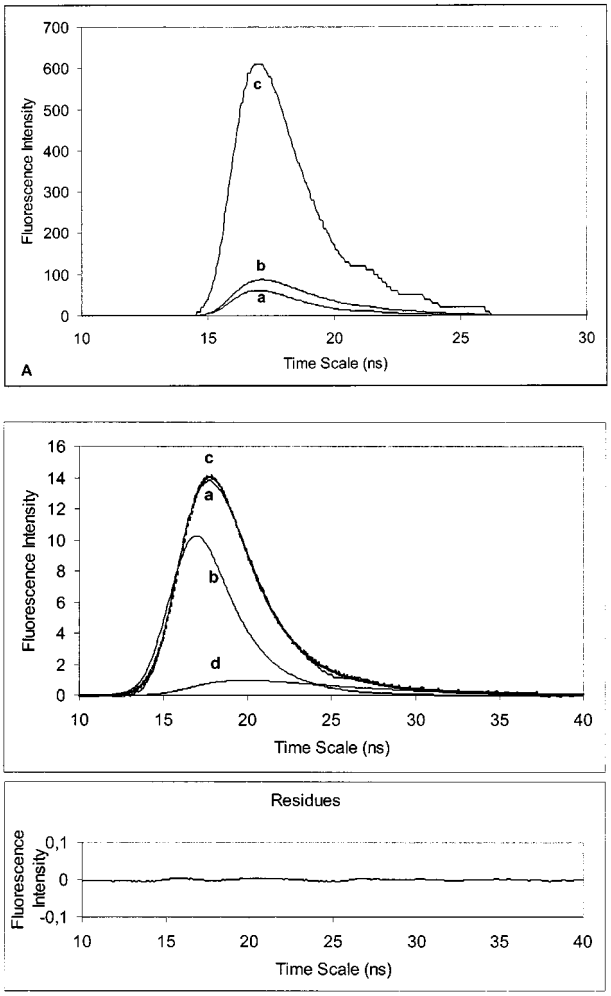

Fig. 4. A: Lifetime measurements: signals issued from a solution 1 μM in 4MeU(Glc)3 alone (curve **a**), in the presence of Tlys (100 μM) (curve **b**), and in the presence of Hlys (100 μM) (curve **c**), respectively. B: Lifetime measurements: resolution of the signal issued from a solution 100 μM in Hlys, 1 μM in 4MeU(Glc)3 (curve **a**): excitation pulse (curve **b**), reconstructed signal (curve **c**), participation of the long time component (t = 6 ns) (curve **c**). The residues (difference between the experimental and the reconstructed curves) are presented below on a larger scale.

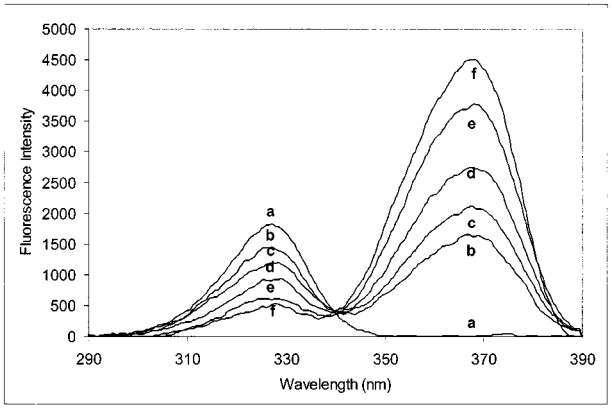

Fig. 5. Changes of the synchronous fluorescence spectrum of a solution 15 μM in Hlys on addition of 4MeU(Glc)3: 0 μM (curve **a**), 2.40 μM (curve **b**), 3.40 μM (curve **c**), 4.90 μM (curve **d**), 7.00 μM (curve **f**), and 8.70 μm (curve **e**).

with their binding to the productive site of Hlys. So it can be assumed that the decrease in the fluorescence intensity of Hlys results from the binding of chitobiosides both to the productive site of Hlys and to the non-productive site. Binding to the non-productive site has been evidenced by crystallographic studies [29]. From what has been previously published, it is obvious that these two complexes must differ only in the way that the substrate is engulfed in the cleft of the protein, suggesting that they must differ by their efficacy in the quenching of the tryptophans that are present in that cleft. This condition was taken into account, specifically, in our model used to get the fit displayed in Fig. 8.

Moreover, such a hypothesis is consistent with the fact that the same kind of complex quenching is observed,

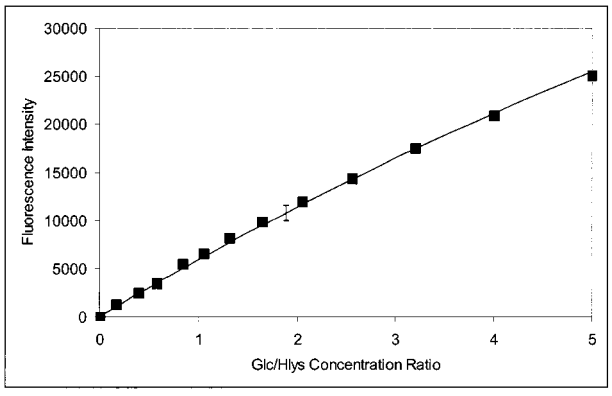

Fig. 6. Modeling changes in the intensity of the synchronous fluorescence of a solution of Hlys (2 μM) on addition of 4MeU(Glc)3: increase of 4MeU(Glc)3 fluorescence intensity. Black squares: experimental results. Curve: theoretical values obtained with a Ka value of 20 μM (Yang and Hamaguchi, 1980).

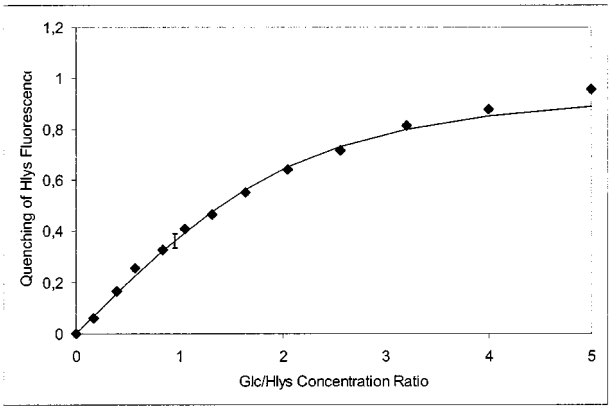

Fig. 7. Modeling changes in the intensity of the synchronous fluorescence of a solution of Hlys (2 μM) on addition of 4MeU(Glc)3: percentage of quenching of the intensity of the synchronous fluorescence spectrum of Hlys. Black squares: Experimental results; curve: theoretical values obtained using two Ka values, 20 μM and 0.4 μM respectively. The quenching efficacy of the productive, less engulfed complex (Ka1 = 20 μM) was taken as 0.85 of that of the non-productive, fully engulfed complex (Ka2 = 0.4 μM).

in the same concentration range, when experiments are performed with Tlys instead of Hlys. But the modeling is more tedious here, for the binding of the chitobiosides to Tlys induced no change in their fluorescence intensity (data not shown).

CONCLUSION

Although previous studies have focussed on the interaction between lysozymes and chitins or fluorescent analogs, some aspects of the biophysical and biological processes remained to be elucidated. It has been well established that chitins bind to the cleft of lysozymes, and the subsites of the locations of the different *N*-acetylglucosamines that are parts of chitins have been identified. Moreover, it has been well documented that a 1,4-β-bond must be located between subsite D and subsite E to be cleaved.

Nevertheless only non-productive associations, in which the fluorescent analogs are located in the A–D subsites, have been detected using crystallographic methods, whereas the existence of productive complexes has been evidenced by catalytic studies. The latter complexes were found responsible for the increase in the fluorescence intensity of the fluorescent analogs of chitobiosides upon binding to Hlys, although no explanation was suggested for the increase.

In this paper, we have first used pulsed fluorescence techniques to study the fluorescence of free and lyso-

zyme-bound 4MeU(Glc)3. After pulsed excitation, the fluorescence of free 4MeU(Glc)3 experienced a fast mono-exponential decay ($\tau = 0.6$ ns.) that suggests that most of the fluorescence is issued from the Frank-Condon state because of a very efficient vibrational deactivation process of the excited states, resulting from the presence of the *N*-acetylglucosamines. In the presence of a large excess of Hlys the fluorescence decay was found to be bi-exponential, with a large increase in the intensity of the fast decay ($\tau = 0.8$ ns) and a small participation of a slow decay ($\tau = 6$ ns). These findings are consistent with a decrease of the freedom of vibrations of the *N*-acetylglucosamines, resulting from their location in the cleft of the protein. As expected, no change was observed in the presence of Tlys, which did not induce increase in the fluorescence intensity of 4MeU(Glc)3.

Synchronous fluorescence techniques were then used to monitor changes in the fluorescence intensity of Hlys, 4MeU(Glc)3, and 4MeU(Glc)2 resulting from additions of substrates of varying concentrations to a solution in which the Hlys concentration was kept constant. It was found that the increase in the fluorescence intensity of the substrates results only from the binding to the productive site while the quenching of the protein fluorescence must result from the binding to both the productive and non-productive binding sites. All previously published papers suggest that the complexes resulting from these bindings differ only by the way that the substrate is engulfed in the protein cleft. The productive complex must be less engulfed than the non-productive one, for the 1,4-β-bond must be located between subsite D and subsite E to be cleaved so that it modifies to a lesser extend the microchemical environment of Trp 62 and Trp 63 of Hlys (see Fig. 2).

Our results suggest therefore that both Hlys-chitobiosides and Tlys-chitobiosides partition in two energetic states separated by a low-energy barrier, resulting in:

- a productive conformation that is responsible for the cleavage of the 1,4-β-bond located between subsite D and subsite E,
- a non-productive conformation, far more populated than the productive conformation at room temperature, in which the substrate is located between the subsites A and D. The non-productive conformation can be considered a "storage conformation" that is detectable, and previously has been detected, through crystallographic studies.

These results suggest that the existence of both of these complexes may give us a tool for probing conformational changes inside the cleft of Hlys and of proteins with similar structures.

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REFERENCES

1. S. Kuramitsu, K. Ikeda, K. Hamaguchi, H. Fujio, T. Amano, S. Miwa, and T. Nishina (1974) *J. Biochem.* **76**, 671–683.
2. T. Imoto, L. N. Johnson, A. C. T. North, D. C. Phiilips, and J. A. Rupley (1972) in P. Boyer (Ed.) *The Enzymes* 3rd ed., Vol. 7, Academic Press, New York, pp. 665–868.
3. K. Hamaguchi, K. Rokkaku, M. Funatsu, and K. Hayashi (1960) *J. Biochem.* **48**, 351–357.
4. K. Hayashi, N. Yamasaki, and M. Funatsu (1964) *Agric. Biol. Chem.* **28**, 517–523.
5. T. Imoto and K. Yagishita (1971) *Agric. Biol. Chem.* **35**, 1154–1156.
6. N. Yamasaki, T. Tsujita, and M. Takakuwa (1973) *Agric. Biol. Chem.* **37**, 1507–1508.
7. R. C. Davies, A. Neuberger, and B. M. Wilson (1969) *Biochim. Biophys. Acta* **178**, 294–305.
8. J. Saint-Biancard, P. Chuzel, Y. Mathieu, J. Perrot, and P. Jolies (1970) *Biochim. Biophys. Acta* **220**, 300–306.
9. P. Maurel and P. Douzou (1976) *J. Mol. Biol.* **102**, 253–264.
10. J. A. Rupley (1964) *Biochim. Biophys. Acta* **83**, 245–255.
11. J. A. Rupley and V. Gates (1967) *Froc. Natl. Acad. Sci. US.* **57**, 496–510.
12. S. K. Banerjee, L. Kregar, V. Turk, and J. A. Rupley (1973) *J. Biol. Chem.* **248**, 4786–4792.
13. S. K. Banerjee and J. A. Rupley (1975) *J. Biol. Chem.* **250**, 8267–8274.
14. T. Osawa and Y. Nakazawa (1966) *Biochim. Biophys. Acta* **130**, 56–63.
15. C. S. Tsai, J. Y. Tang, and S. C. Subbarao (1969) *Biochem. J.* **114**, 529–534.
16. T. Rand-Mejr, F. W. Dahlquist, and M. A. Raftery (1969) *Biochemistry* **8**, 4206–4214.
17. F. W. Ballardie, B. Capon, M. W. Cuthbert, and W. M. Dearie (1977) *Bioorg. Chem.* **6**, 483–509.
18. N. Sharon (1967) *Proc. Roy. Soc.* **B167**, 402–415.
19. D. M. Chipman, J. J. Pollock, and N. Sharon (1968) *J. Biol. Chem.* **243**, 487–496.
20. J. A. Rupley (1967) *Proc. Roy. Soc.* **B167**, 416–428.
21. N. A. Kravchenko (1967) *Proc. Roy. Soc.* **B167**, 429–430.
22. J. J. Pollock and N. Sharon (1970) *Biochemistry* **9**, 3913–3925.
23. M. O'Brien and R. R. Colwell (1987) *Appl. Environ. Microbiol.* **53**, 1718–1720.
24. M. Hood (1991) *J. Microbiol. Methods* **13**, 151–160.
25. Y. Yang, and P. L. Hamaguchi (1980) *J. Biochem.* **87**, 1003–1014.
26. S. Karsen and E. Hough (1995) *Acta Cryst.* **D51**, 962–978.
27. S. Karsen and E. Hough (1996) *Acta Cryst.* **D52**, 115–123.
28. S. Karsen, B. E. Eliassen, L. K. Hansen, R. L. Larsen, B. W. Riize, A. O. Smalas, E. Hough, and B. Grinde (1995) *Acta Cryst.* **D51**, 354–367
29. V. B. Vollan, E. Hough, and S. Karlsen (1999) *Acta Cryst.* **D55**, 60–66.
30. C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma (1967) *Proc. Roy. Soc.* **B167**, 378–388.
31. T. Vo-Dinh (1978) *Anal. Chem.* **50**, 396–401.