A SPECTROFLUOROMETRIC STUDY OF TRYPTOPHAN 108 IN HEN EGG-WHITE LYSOZYME

Vivian I.TEICHBERG and Nathan SHARON
Department of Biophysics, The Weizmann Institute of Science
Rehovoth, Israel

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1. Introduction

The molecule of hen egg-white lysozyme contains six tryptophan residues, three of which (Trp 62, 63, 108) have been located by X-ray crystallography in the region of the binding site of saccharides to enzyme [1]. Upon interaction with substrates or inhibitors in solution, the tryptophan residues are perturbed as reflected by changes in their UV [2, 3], CD [4] and fluorescence emission spectra [5, 6]. Using difference fluorescence methods, Lehrer and Fasman [6] separated qualitatively the contributions of the different active site tryptophans. They concluded that two of these tryptophan residues, possibly Trp 62 and Trp 63, are in an aqueous environment in the free enzyme and in a less aqueous environment in the enzyme-inhibitor ($(GlcNAc)_2$ or $(GlcNAc)_3$) complex. Furthermore, they postulated that Trp 108 interacts with Glu 35 and that weakening of this interaction on binding $(GlcNAc)_3$ is responsible for the increase of the quantum yield of the lysozyme-$(GlcNAc)_3$ complex observed upon changing the pH from 5.5 to 8.

In this communication we present data on the quantitative contribution of Trp 108 to the fluorescence of lysozyme. We show that modification of Trp 108 does not affect the binding of $(GlcNAc)_3$ to lysozyme and conclude that this residue is involved in a change in conformation of the enzyme occurring upon binding of $(GlcNAc)_3$. We also confirm the suggestion [6] that a change in the state of Trp 108 on binding $(GlcNAc)_3$ to the enzyme is responsible for the enhancement of the fluorescence of the lysozyme-$(GlcNAc)_3$ complex in the pH 5.5 to 8 region.

2. Materials and methods

Hen egg-white lysozyme (Worthington, LY 8 HB) was used as purchased. $(GlcNAc)_3$ was isolated by the method of Rupley [7]. Enzymatically inactive iodine oxidized lysozyme (IL) in which only Trp 108 is modified by conversion of its indole moiety to oxindole, was prepared according to Hartdegen and Rupley [8]. Fluorescence measurements were routinely performed with an Aminco-Bowman spectrofluorometer equipped with a cell jacket thermostated at 23°C. Quantum yield measurements were done on a laboratory built spectrofluorometer (Department of Photochemistry, Weizmann Institute of Science). The exciting light was 285 nm. The fluorescence of a reference solution of *p*-terphenyl in cyclohexane [9] was measured at the same time as that of the protein solutions for the determination of quantum yields and for correcting the small instrumental fluctuations. All spectra were corrected for absorbance, so that areas under emission peaks are proportional to quantum yield.

Association constants were determined by fluorometric titrations as described by Chipman et al. [10].

3. Results and discussion

Fig. 1 shows the spectra of lysozyme (a), of IL (b), and IL in the presence of a saturating concentration of $(GlcNAc)_3$ (c). The spectral characteristics, emission λ_{max} and fluorescence quantum yields, are summarized in table 1. The modification of Trp 108 (into oxindole absorption λ_{max} 250 nm [8]) by oxidation of lysozyme is reflected by a dramatic change in the emission spectrum of the enzyme. The fluorescence quantum yield drops by 56% and this drop is accompanied by a shift

Table 1
Effect of iodine oxidation and binding of $(GlcNAc)_3$ on the fluorescence spectrum of hen egg-white lysozyme.

System	λ_{max}(nm)	Q%	K_a (M^{-1})
a. Lysozyme	342	5.62	
b. Iodine oxidized lysozyme	350	2.48	
c. Iodine oxidized lysozyme + $(GlcNAc)_3$	343	2.06	2.1×10^5
d. Trp 108 (by difference a–b)	340	(19)	
e. Trp 62 + Trp 63 (by difference b–c)	360	(1.05)	
f. Lysozyme + $(GlcNAc)_3$	333	5.62	1.9×10^5
g. L-tryptophan	360	20	

Wavelength of maximum fluorescence in NaCl 0.2 M, pH 5.6, 23°C. All protein solutions had an absorbance at 285 nm of less than 0.1. The inhibitor $(GlcNAc)_3$ was at a final concentration of 1 mg/ml. The quantum yields (Q expressed in percent) were determined by comparing the area under the corrected fluorescence spectra of the solutions measured with a reference solution of *p*-terphenyl in cyclohexane, Q (*p*-terphenyl) = 0.92 [9]. The values in parenthesis were calculated by assuming an equal absorbance of all Trp while considering a 56% contribution of Trp 108 to the total fluorescence and a minimum of 17% quenching of Trp 62 and 63 by $(GlcNAc)_3$. The latter figure is the decrease in fluorescence of Trp 62 and 63 resulting from binding of $(GlcNAc)_3$ to IL (see *e*), and may represent partial or complete quenching of these tryptophans. All solutions were excited at 285 nm. The association constants K_a were measured at 325 nm according to the procedure of Chipman et al. [11].

in the fluorescence maximum from 342 to 350 nm. This shift to the red is due to the destruction of a "blue" tryptophan [11]. The spectrum of the tryptophan residue modified by the iodine treatment (Trp 108) is the difference spectrum between lysozyme and IL (fig. 1, d). The resultant curve exhibits a λ_{max} at 340 nm which is the same as found for free tryptophan in methanol. Interestingly, Trp 108 contributes 56% of the total fluorescence of the enzyme, whereas the five other tryptophans together contribute the remaining 44%.

When $(GlcNAc)_3$ is added to IL, a shift in the fluorescence maximum of 7 nm, from 350 to 343 nm, is observed as well as a drop of 17% in the quantum yield (fig. 1, c and table 1). The resultant difference spectrum (fig. 1, d) has a maximum at 360 nm, similarly to free tryptophan in water, showing that the quenched tryptophans are in a polar environment. From the quenching observed upon addition of $(GlcNAc)_3$ to IL, the association constant K_a of the inhibitor-IL complex was calculated as 2.1×10^5 M^{-1} at pH 5.1 and 23°C. The fact that this association constant is, within the limits of error, identical to that found for the binding of $(GlcNAc)_3$ to lysozyme (K_a = 1.9×10^5 M^{-1}) strongly suggests that no major conformational changes, at least in subsites *A*, *B* and *C* of the active site of lysozyme, occur upon iodine oxidation. From the crystallographic model of the lysozyme-$(GlcNAc)_3$ complex, possible interactions have been indicated between the sugar residue in subsite *C* and Trp 108, mainly through the atoms of the five-membered ring of the indole moiety [12]. Modifications of this five-membered ring into oxindole, as mentioned, or into *N*-formyl kyrurenine [13] do not alter the binding properties of the modified enzyme. Very recently, applications of proton magnetic resonance [14], have shown that the indole N*H* proton resonance assigned to Trp 108 was not perturbed by the binding to lysozyme of GlcNAc and $(GlcNAc)_2$. We can therefore safely assume that Trp 108 is not involved in the binding of saccharides to the enzyme.

The quenching and the blue shift resulting from the binding of $(GlcNAc)_3$ to IL can be accounted for by a direct interaction through hydrogen bonds [1] of $(GlcNAc)_3$ with either Trp 62 or Trp 63 or with both. Our experiments do not permit the unambiguous separation of the individual contributions of Trp 62 and Trp 63 to the total fluorescence of the enzyme or to the quenching mechanism. Is it possible that

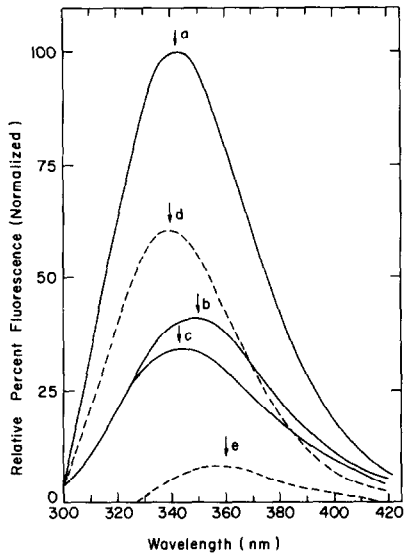

Fig. 1. Fluorescence spectra in 0.2 M NaCl, pH 5.6. 23°C. *a*, native lysozyme; *b*, iodine oxidized lysozyme; *c*, iodine lysozyme + 0.1% (GlcNAc)3; *d*, difference fluorescence spectrum, from a–b; *e*, difference fluorescence spectrum from *b*–*c*. The arrows indicate the position of the fluorescence maximum. Protein concentration, 0.005%, excitation at 285 nm. 1 cm path length cell.

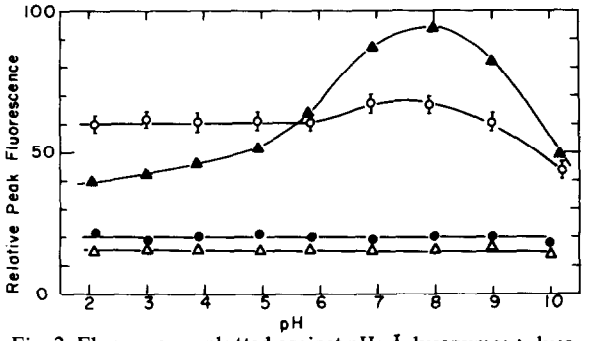

Fig. 2. Fluorescence plotted against pH: ◊, lysozyme; ▲, lysozyme + 0.1% (GlcNAc)3; ●, iodine oxidized lysozyme; △, iodine oxidized lysozyme + 0.1% (GlcNAc)3. t = 23°C. Excitation at 285 nm, 1 cm path length. Lysozyme emission measured at 340 nm; Lysozyme + (GlcNAc)3 at 335 nm; iodine oxidized lysozyme at 350 nm; iodine oxidized lysozyme + (GlcNAc)3 at 345 nm. Protein concentration, 0.005%. Buffers: pH 2.2 to 3.6, 0.05 M glycine-HCl; pH 4 to 5.6, 0.1 M sodium acetate; pH 6 to 8, 0.1 M sodium phosphate; pH 8.6 to 10.6, 0.05 M glycine-NaOH. No influence of buffer ions on the emission spectrum was observed.

energy transfer takes place between Trp 62 and Trp 63 so that no separation of the fluorescence contribution of each of these residues [15] can be made.

As seen in table 1, the fluorescence maximum of the lysozyme-$(GlcNAc)_3$ complex (at pH 5.6) is at 333 nm which is lower than that of free enzyme (342 nm) suggesting that upon formation of the complex not only do Trp 62 and 63 pass from a hydrophilic environment (360 nm) to a hydrophobic environment (343 nm) but also Trp 108 passes to a more hydrophobic environment than it occupies in the native protein. As we have concluded that Trp 108 was not involved in the binding of $(GlcNAc)_3$, the change in its environment can only result from a local change in conformation occurring on binding $(GlcNAc)_3$ in the active site.

Fluorometric titrations of IL and of its $(GlcNAc)_3$ complex with hydroxide ions show that in both cases, the fluorescence is independent of pH, and that no enhancement is observed in the pH 5.5 to 8 region (fig. 2). Since the iodine treatment is known to affect only Trp 108 [8], we can unambiguously assign this residue as being the tryptophan involved in the enhancement of the fluorescence of the lysozyme-$(GlcNAc)_3$ observed in the pH 5.5 to 8 region. Lehrer and Fasman [6] have suggested that the titration of a carboxylate ion of high p*K*, most probably Glu 35, (p*K* 6.3), is responsible for this enhancement. From a set of improved coordinates of the atoms of lysozyme based on the model of Phillips [1], a distance of 4.9 Å was calculated (F.Saya, private communication), between the hetero nitrogen of the indole ring of Trp 108 and one of the carboxyl oxygen atoms of Glu 35. Similar calculations have also shown that this distance is the closest distance found between any ionized group and any tryptophan of the enzyme. Since carboxyls are known to quench tryptophan fluorescence, depending on the distance between the quencher and the indole ring [16], the results of the fluorometric titration of lysozyme and its $(GlcNAc)_3$ complex can be interpreted as follows: In the native enzyme, Trp 108 is quenched by Glu 35 whose carboxyl oxygen lies very close to the indole ring. Upon binding of $(GlcNAc)_3$ a change in conformation takes place pulling Glu 35 apart from Trp 108. The enhancement of the fluorescence of the enzyme in the pH 5.5 to 8 region reflects this change in conformation. Assuming that the conformation of the productive enzyme-substrate complex is the same or similar to that of the non-productive

lysozyme-$(GlcNAc)_3$ complex, then this conformation may be more favorable for catalysis by bringing Glu 35 into an optimal position for protonation and cleavage of the glycosidic bond [17].

References

[1] D.C.Phillips, Sci. Am. 215 (5) (1966) 78; Proc. Natl. Acad. Sci. U.S. 57 (1967) 48.
[2] K.Hayashi, T.Imoto and G.Funatsu, J. Biochem. Tokyo, 55 (1964) 516.
[3] F.W.Dahlquist, L.Jao and M.Raftery, Proc. Nat. Acad. Sci. U.S. 56 (1966) 26.
[4] A.N.Glazer and N.S.Simmons, J. Am. Chem. Soc. 88 (1966) 2335.
[5] M.Shinitzki, V.Grisaro, D.M.Chipman and N.Sharon, Arch. Biochem. Biophys. 115 (1966) 232.
[6] S.S.Lehrer and G.D.Fasman, Biochem. Biophys. Res. Commun. 23 (1966) 133; J. Biol. Chem. 242 (1967) 4644.
[7] J.A.Rupley, Biochim. Biophys. Acta 83 (1964) 245.
[8] F.J.Hartdegen and J.A.Rupley, Biochim. Biophys. Acta 92 (1964) 625; F.J.Hartdegen, Ph.D.Thesis, University of Arizona 1967.
[9] I.B.Berlman, Handbook of fluorescence spectra of aromatic molecules (Academic, 1965).
[10] D.M.Chipman, V.Grisaro and N.Sharon, J. Biol. Chem. 242 (1967) 4388.
[11] Y.Elkana, J. Phys. Chem. 72 (1968) 3654.
[12] W.J.Browne, A.C.T.North, D.C.Phillips, Keith Brew, T.C. Vanaman and R.L.Hill, J. Mol. Biol. (1969) 42, 65.
[13] A.Previero, M.A.Coletti-Previero and P.Jollès, J. Mol. Biol. 24 (1967) 261.
[14] J.D.Glickson, C.C.McDonald and W.D.Phillips, Biochem. Biophys. Res. Commun. 35 (1969) 492.
[15] J.W.Longworth, Biopolymers 4 (1966) 1131.
[16] R.W.Cowgill, Arch. Biochem. Biophys. 100 (1963) 36.
[17] J.J.Pollock, U.Zehavi, V.I.Teichberg and N.Sharon, Israel J. Chem. 6 (1968) 112p.