Kinetic and Equilibrium Studies of Cyclodextrin and Amino Acid-Thiol-o-Phthalaldehyde(OPA) Reaction using Flow Injection Analysis and Fluorescence Detection

[1]MUHAMMAD YOUNAS KHOKHAR AND [2]J.N. MILLER
[1]*Department of Chemistry, Bahauddin Zakariyia University, Multan, Pakistan*
[2]*Department of Chemistry, Loughborough University of Technology, Loughborough, Leicestershire, LE11 3TU, U.K.*

(Received 5th December, 1994, revised 3rd June, 1995)

Summary: The effect of cyclodextrin on the fluorescence intensity of amino acid-thiol-OPA reaction has been studied. The host-guest inclusion phenomenon was successfully observed for DL-lysine and L-serine with cyclodextrins. Fluorescence enhancement was in agreement with expectations. Kinetic and equilibrium studies were made by flow injection fluorescence analysis. Effect of thiol and time on fluorescence intensity were recorded.

Introduction

Cyclodextrins (CyD) are cyclic, water soluble receptor compounds. The most common are α-, β- and γ-cyclodextrins. They forms inclusion complexes, with the characteristic structure of an adduct and compounds of a size compatible with the dimensions of the cavity. The interaction between substrate and cyclodextrin depends on solute size and geometry. Van der Waals interactions, hydrogen bonding, release of strain energy in the cyclodextrin ring and release of high energy water molecules from the cyclodextrin cavity are also involved. Various influences of cyclodextrin inclusion complexes on guest molecules have been reported, for examples, sheltering of hidden parts and enhanced reactivity of exposed parts of the guest molecules, and strong interactions within a guest pair caged in the cyclodextrin cavity. Inclusion complexes alter many properties of the included molecule, for example, chemical reactivity, volatility, and absorption, fluorescence and circular dichroism. These changes in chemical and physical properties are of both theoretical and practical interest.

Ortho-phthalaldehyde (OPA) reacts with amino acids in the presence of a strong reducing agent, i.e. a thiol and in aqueous alkaline solution (pH- 8-10) to form a fluorescent isoindole product [1]. This reaction has been widely used as a labelling agent for amino acids and proteins in both pre- [2,3] and post-column [4,5] derivatisation chromatographic methods. The addition of cyclodextrin changed the fluorescence [6-8] and UV-visible spectra [9,10] of various compounds. Only a few kinetic [11,12] and equilibrium [13-14] studies have been reported in the literature of various reactions. This paper describes the use of flow injection analysis in the determination of kinetic and equilibrium studies of the host-guest complex formation and the effects of CyDs on the fluorescence intensities and stabilities of Amino acid-Thiol-OPA reaction.

Results and Discussion

Effect of cyclodextrins on fluorescence intensity

In the past cyclodextrins have been used to enhance the fluorescence intensity and improve the stability of many compounds by forming inclusion complexes. The addition of β-cyclodextrin to fluorigenic reagent (OPA-2-ME or ethanethiol) and DL-lysine enhances the fluorescence intensity in

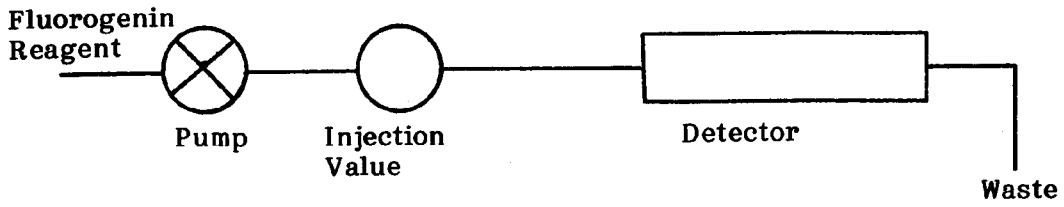

Fig.1: Flow injection manifold for the fluore-scence enhancement of OPA-Thiol-DL-lysine or L-serine with cyclodextrin.

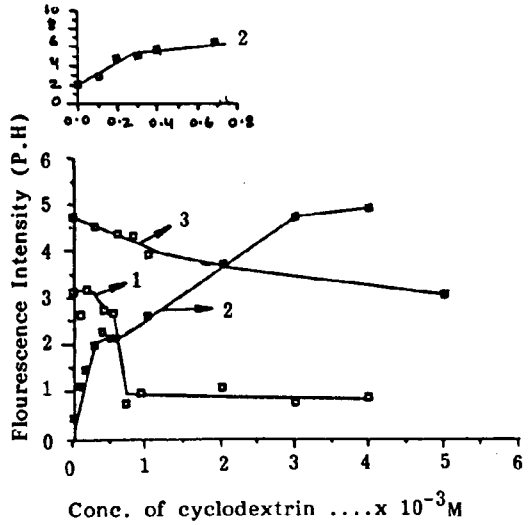

Fig.2: Fluorescence intensity (Peak Height) vs conc. of cyclodextrin of OPA-2-ME-DL-lysine in FIA system (1=α, 2=β,3=γ-cyclodextrin).

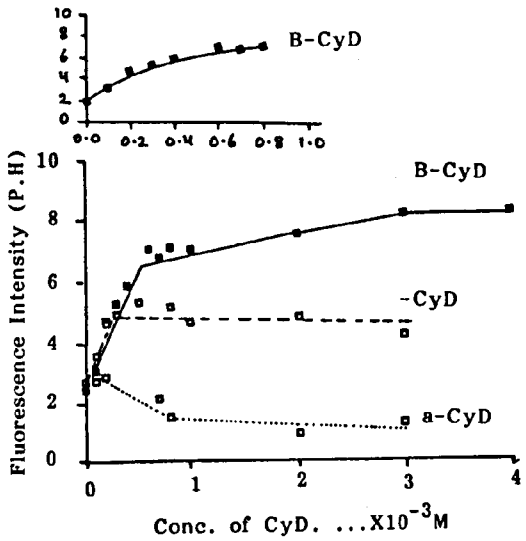

Fig.3: Fluorescence intensity (Peak Height) vs conc. of cyclodextrin of OPA-ethanethiol-DL-lysine in FIA system.

two steps (Figs. 2,3). Enhancement was first observed with a β-cyclodextrin concentration of 10^{-4}M and continued enhancement occurred up to $3x10^{-4}$Mβ-CyD. While the second fluorescence enhancement was observed from 10^{-3} to $3x10^{-3}$M β-CyD concentration. The isoindoles of DL-lysine are too large to be completely included into a single β-CyD cavity. It can accommodate only one isoindole group leaving the other exposed to solvent molecules (Fig. 4). At higher concentration, additional cyclodextrin molecules associate together to form a 2:1 complex and hence incorporates the both isoindoles into the β-CyD cavities. γ-Cyclodextrin has larger cavity [15]. It appears that one of the isoindole formed from the reaction of OPA-ethanethiol-DL-lysine incorporates into the γ-cyclodextrin cavity and shows enhancement as the concentration of γ-cyclodextrin was increased from 10^{-4}M to $3x10^{-4}$M. Further addition of γ-CyD did not enhance the fluorescence intensity. It can be said that the second isoindole of the reaction OPA-ethanethiol-DL-lysine does not fit into the γ-cyclodextrin cavity. Hence no complexation formed with the second isoindole group and therefore, no increase in fluorescence intensity was observed with further addition of γ-cyclodextrin (Fig. 3). The fluorescence enhancement was also observed for the isoindole of OPA-2-ME-L-serine with the addition of γ-cyclodextrin.

There was no fluorescence enhancement observed for α-cyclodextrin. The fluorescence of the isoindole of DL-lysine was rather quenched by an increased concentration of α-CyD (Figs. 2,3).

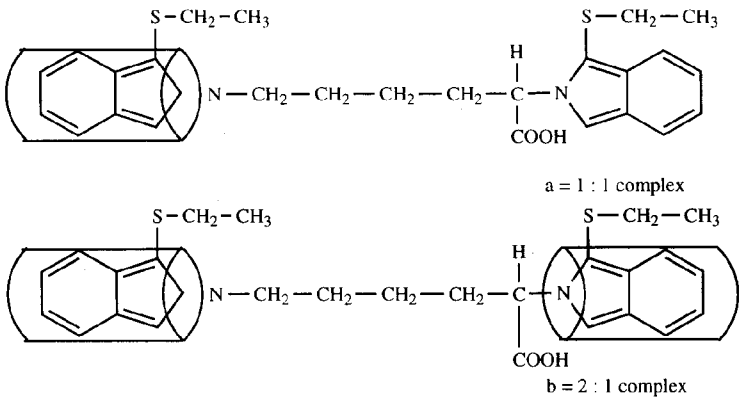

Fig.4: Binding of β-cyclodextrin to the isoindole of DL-lysine.

The cavity of α-cyclodextrin is too small to form an inclusion complex with the isoindole of DL-lysine and L-serine, hence no fluorescent enhancement was observed.

The mechanism for the formation of an inclusion complex was explained by Bender *et al.* [16,17] and Bergeron *et al.* [18,19] using thermodynamic parameters in terms of the release of high energy water from the cyclodextrin cavity. The water molecules associated with the cavity are enthalpy rich, as they cannot have a full compliment of hydrogen bonds, due to interference from the glycopyranose rings of cyclodextrin. The inclusion of substrate results in the expulsion of these water molecules into the surrounding system. Saenger [20,21] found using X-ray crystallographic data that the molecules of α–CyD have an unstrained hexagonal geometry in most α-CyD adducts, except for the water-α-CyD adduct where the macrocyclic conformation of α-CyD is unsymmetrically distorted and energetically unstable. Tabushi [22] showed that strain release, release of high energy water molecules, Van der Waals interactions and breaking of water clusters around an apolar guest molecule are the important forces stabilising the inclusion complex.

Effect of Thiol

A thiol was essential for the reaction between OPA and amino acids. The two step fluorescence enhancement observed for β-CyD and isoindole of DL-lysine was found with 2-mercaptoethanol and ethanethiol (Fig. 2,3), but not with 2-methyl-2-propanethiol (Fig. 5). With γ-CyD ethanethiol gave a single step enhancement (Fig. 3). The 2-methyl-2-propanethiol is a large molecule compared to 2-mercaptoethanol and ethanethiol. As a result of its size the isoindole of DL-lysine could not center the β- or γ-CyD cavity and hence no enhancement was observed.

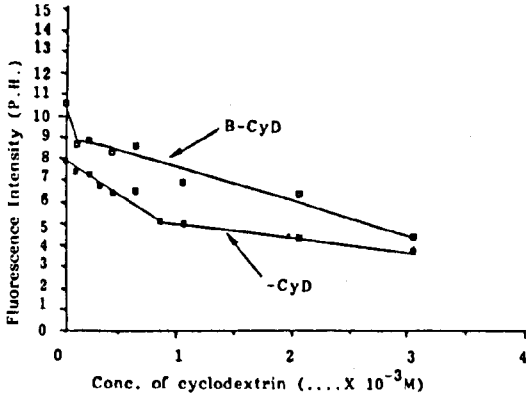

Fig.5: Fluorescence intensity (Peak Height) vs conc. of cyclodextrin of OPA-2-methyl,2-propanethiol-DL-lysine in FIA system.

The addition of γ-CyD to the isoindole of L-serine formed in the presence of 2-mercaptoethanol (i.e; OPA+2+ME+L-serine+γ-cyclodextrin) and not ethanethiol (i.e; OPA+ethanethiol+L-serine+γ-cyclodextrin), showed increase of fluorescence. It is probable that hydrogen bonding inside the cavity of γ-cyclodextrin is more favourable for 2-mercaptoethanol than for the ethanethiol complex, as the former has an -OH group available to participate in hydrogen bonding.

The observed enhancement were by factors of 2- to 10-fold. These results point to a combination of factors being responsible for the fluorescence enhancement, with specific interaction effects as well as the size compatibility of the cyclodextrin cavity and the isoindole influencing the outcome.

Dependence of absorption and emission

Wavelength

As shown in Tables 1,2,3 the emission wavelength shifts towards a shorter wavelength and the absorption wavelength shifts towards a longer wavelength with the addition of cyclodextrins. A maximum of ten nanometer blue shift was observed in the fluorescence spectra, while only one to four nanometer red shift was observed in the UV spectra. Fluorescence enhancement due to the addition of γ-cyclodextrin was more than the absorption one. The blue shift in fluorescence spectra and red shift in absorption spectra is a characteristic feature of inclusion complexes.

Table-1: Maximum emission wavelength of OPA-Thiol-DL-Lysine and OPA-Thiol-L-Serine with and without cyclodextrin, excitation wavelength = 337 nm.

Thiol	Dl-Lysine α-CyD	β-CyD	γ-CyD	L-serine α-CyD	β-CyD	γ-CyD	λem(nm)
2-Mercap	-	-	-				453
toethanol	+	-	-				454
	-	+	-				446
	-	-	+				456
				-	-	-	449
				-	-	+	451
Ethane	-	-	-				458
thiol	+	-	-				460
	-	+	-				449
	-	-	+				452

Table-2: Maximum absorption wavelenght of OPA-Thiol-Dl-Lysine with and without cyclodextrin (CyD)

Thiol	Wavelength (nm) No CyD	β-CyD	γ-CyD
2-Mercaptoethanol	291	293 at $3x10^{-4}$M	-
	333	290 at $3x10^{-3}$M	
Ethanethinol	291	293 at $3x10^{-4}$M	291
	333	290 at $3x10^{-3}$M	336

Table-3: Maximum absorption wavelength (nm) of OPA-Thiol-L-serine with and without cyclodextrin (CyD)

Thiol	No CyD	γ-CyD
2-Mercaptoethanol	282	282
	332	335

Influence of the Thiol structure on stability

The stability curves for OPA-ethanethiol-DL-lysine with and without β-cyclodextrin are shown in Fig. 6. The figure showed that they are not stable. The stability curves for β-cyclodextrin with OPA-2-mercaptoethanol-DL-lysine and γ-cyclodex-trin with OPA-ethanethiol-DL-lysine and OPA-2-mercaptoethanol-L-serine were also found unstable. It is possible that the bonding inside the cavity of cyclodextrin were not strong enough to stabilise the complexes.

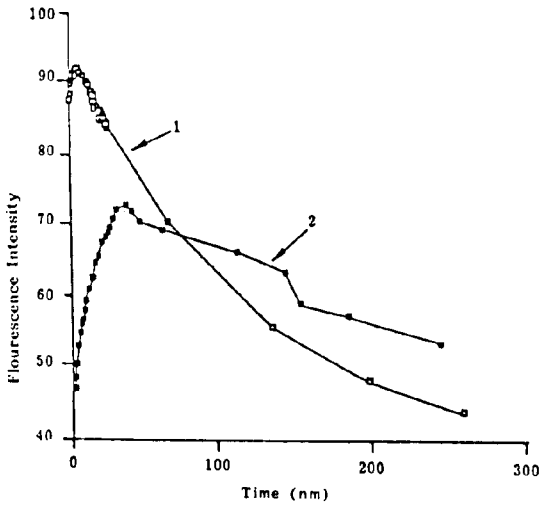

Fig.6: Fluorescence intesntiy vs time of OPA + ethanethiol + DL-lysine with (1) and without (2) β-cyclodextrin. Excitation wavelength filter = 337 nm, λem = 450 nm.

Rate and formation constants

Rate and formation constants with and without cyclodextrin and OPA-thiol-DL-lysine and OPA-thiol-L-serine are shown in Table-4 and 5. A graphical method was used to calculate the rate constants. All reactions with and without cyclodextrin were observed to be first order. The rate constant with cyclodextrin was found to be higher than without it. Generally rates are higher in an apolar medium than polar. The cyclodextrin cavity provides an apolar environment to its guest by expelling the water from its cavity.

The formation constants for cyclodextrin complexes were calculated using the Benesi and Hildebrand equation [23].

$$Ca/\Delta I = 1/K\Delta i.1/Cd + 1/\Delta i$$

where Ca represents the concentration of amino acids, Cd = concentration of cyclodextrin, ΔI= increment of fluorescence of OPA-thiol-DL-lysine on the addition of the cyclodextrin, and ΔI = ΔI/conc. of OPA-thiol-DL-lysine-CyD complex.

Table-4: Association and rate constants of OPA-Thiol-DL-lysine with and without cyclodextrin (CyD).

Host	Guest	Association constants		Rate constants	
		K'	K''	$K1_{min^{-1}}$	$K2_{min^{-1}}$
-	OPA-Ethanethiol-DL-Lysine	-	-	32.242	-
-	OPA-2-ME-DL-Lysine	-	-	13.818	-
β-CyD	OPA-2-ME-DL-Lysine	$3.072x10^3$	$4.673x10^2$	16.889	15.7445
β-CyD	OPA-Ethanethiol-DL Lysine	$3.265x10^3$	$1.548x10^3$	37.9995	13.48
γ-CyD	OPA-Ethanethiol-DL-Lysine	$3.7015x10^3$	-	21.8179	-

Table-5: Association and rate constants of OPA-Thiol-L-serine with and without cyclodextrin (CyD)

Host	Guest	Association constant	Rate constant
		K'	$K1_{min^{-1}}$
-	OPA-2-ME-L-serine	-	0.7816
γ-CyD	OPA-2-ME-L-serine	8.255910^5	1.336

Other isoindole-cyclodextrin complexation

Enhancement of the fluorescence signals of other OPA-thiol-amino acid products with cyclodextrin was also investigated. Glycine, aspartic acid, tyrosine, glutamic acid, and alanine were investigated. Initially, fluorescence enhancement was observed for glycine, aspartic acid and tyrosine under the same conditions as that for DL-lysine and L-serine, but not for glutamic acid and alanine. Later experiments showed no enhancement for glycine, aspartic acid and tyrosine as well. Experimental conditions like pH, flow rate, concentration of amino acids, time of reaction, etc. were varied to achieve the fluorescence enhancement with cyclodextrins, but in vain. It seemed that many factors are responsible for the fluorescence enhancement, with specific interaction effects as well as size compatibility of CyD cavity and the isoindole influencing the outcome.

Experimental

Materials

α-, β- and γ-Cyclodextrins, OPA and 2-mercaptoethanol (2-ME), were purchased from Sigma. While ethanol, sodium tetraborate were from Fisons and L-serine, ethanethiol and 2-methyl, 2-propanethiol were provided by Aldrich. DL-Lysine. HCl was supplied by the Hopkin and William. All solutions were prepared in water (0.05 μS/cm) purified using a Liquipure Modulab System.

Methods

Fluorigenic reagents

Ortho-phthalaldehyde (0.2 g) was dissolved in 2.00 ml of ethanol Then 50 μl of thiol, i.e. 2-mercaptoethanol or ethanethiol, was added and the total volume was made up to 250 ml with 0.025 M $Na_2B_4O_7$ pH 9.26.

Cyclodextrin solution

A stock solution was prepared every day by dissolving an appropriate quantity of cyclodextrin in the fluorigenic reagent solution using ultrasonication. The standard and other cyclodextrin solutions were prepared from the stock solution by taking an appropriate volume of stock solution and diluting it with fluorigenic reagent solution.

Amino acid solution

Amino acid solutions such as DL-lysine, L-serine, glycine, tyrosine, alamine, aspartic acid and glutamic acid, were 10 μmol/l except L-serine which was 1 μmol/l. An appropriate quantity of them was weighed and dissolved in distilled water.

Instrumentation

The flow injection fluorescence measurements were carried out on a Perkin-Elmer LS-2B Filter Fluorimeter fitted with a flow cell of 7 μl illuminated volume. It was connected to an X-Y chart recorder to record the time drive. While the static UV-visible and fluorescence spectra were recorded on Pye Unicam UV-visible spectrophotometer PU8600 and Perkin Elmer LS-5

Luminescence Spectrometer connected to a Luminescence Data Station.

Procedure

The fluorigenic reagent with or without cyclodextrin was continuously pumped (3.4 ml/min) through the manifold (Fig. 1). The amino acid solutions were injected into the carrier stream using an injection valve (injected volume 75 µl). The amino acid solutions mixed and reacted with the carrier stream while moving towards the detector, where fluorescence was continuously recorded. UV and fluorescence spectra were measured statically in 3 ml fluorescence cells.

References

1 M. Roth, *Anal. Biochem.*, **43**, 880 (1971).
2. B.N. Jonnes, S. Paabo, S. Stein, *J. Liq. Chromatog.*, **4**, 565 (1981).
3. M. Griffin, S.J. Price, T. Palmer, *Clin. Chim. Acta*, **125**, 89 (1982).
4. P.C. Jocelyn, A. Kamminya, *Anal. Biochem.*, **37**, 417 (1970).
5. D.H. Spackman, S. Moore, W.H. Stein, *Anal. Chem.*, **30**, 1190 (1958).
6. G. Patonay, M.E. Rollie, I.M. Warner, *Anal.Chem.*, **57**, 569 (1985).
7. S. Scypinski, L.J.C. Love, *Anal. Chem.*, **56**, 322 (1984).
8. M. Kempfle, R. Mueller, R. Palluk, Z. *Anal.Chem.*, 317, 700 (1984).
9. Kodaka, T. Fukaya, *Bull. Chem.Soc. Jpn.*, **59** 2032 (1986).
10. S.F. Lincoln, A.M. Hounslow, J.H. Coates, B.J. Doddridge, *J. Chem. Soc. Faraday Trans.I*, **83**, 2697 (1987).
11. R.L. Schiller, S.F. Lincoln, J.H. Coates, *J. Chem. Soc. Faraday Trans. I*, **82**, 2123 (1986).
12. R.L. Schiller, S.F. Lincoln, J.H. Coates, *J. Inclusion Phenom.* **5**, 59 (1987).
13. G. Nelson, G. Patonay, I.M. Warner, *J. Inclusion Phenom* **6**, 277 (1988).
14. M. Fujiki, T. Deguchi, I. Sanemasa, *Bull. Chem. Soc. Jpn.*, **61**, 1163 (1988).
15. M.A. Parrish, *Spec. Chem.*, 7(6), 366 (1987).
16. D.W. Griffiths, M.L. Bender, *Adv. Catal.*, **23**, 209 (1973).
17. R.L. VanEtten, J.F. Sebastian, G.A. Clowes, M.L. Bender, *J.Am.Chem.Soc.*, **89**, 3242 (1967).
18. R.J. Bergeron, M.A. Channing, G.J. Gibeily, D.M. Pillor, *J.Am.Chem.Soc.*, **99**, 5146 (1977).
19. R.J. Bergeron, M.P. Meeley, *Bioorg. Chem.*, **5**, 197 (1976).
20. P.C. Manor, W. Saenger, *J.Am.Chem.Soc.*, **96**, 3630 (1974).
21. W.Saenger, R.K. McMullan, J. Fayos, D. Mootz, *Acta Crystallogr. Sect.B* **30**, 2019 (1974).
22. I. Tabushi, Y. Kiyosuke, T. Sugimoto, K.Yamamura, *J. Am.Chem.Soc.*, **100**, 916 (1978).
23. H.A. Benesi, J.H. Hildebrand, *J.Am.Chem. Soc.*, **71**, 2703 (1970).