ENHANCEMENT OF THE FLUORESCENCE INTENSITY OF DANSYL AMINO ACIDS IN AQUEOUS MEDIA AND ITS APPLICATION TO ASSAY OF AMINO ACIDS

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Summary: The fluorescence of dansyl amino acids in aqueous media was found to be greatly enhanced by addition of cycloheptaamylose. This finding was successfully applied for improvement of the assay procedure of amino acids. Interaction of cycloheptaamylose with dansyl amino acids was thermodynamically studied. The entropies and the enthalpies were found to vary with polarities of the dansyl amino acids.

Microquantities of biologically active amino compounds such as amino acids, peptides and catecholamines are currently determined by the use of dansyl chloride (1, 2). Dansyl derivatives of these compounds give poor fluorescence in aqueous media (3). Therefore, the compounds dansylated in the aqueous solution must be transferred to non-polar media prior to measurement of the fluorescence (4). This procedure is time-consuming and inapplicable to dansyl derivatives insoluble in non-polar solvents. In the present study, cycloheptaamylose (C7A) was found to greatly enhance the fluorescence intensity of various dansyl derivatives in aqueous media. An application of this finding to the determination of amino acids in aqueous media and some thermodynamic studies on the interaction of C7A are also described below.

Abbreviations: dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; C7A, cycloheptaamylose (β-cyclodextrin). In figures and tables are used the following abbreviations for dansyl derivatives (1): DANS-OH, dansylsulfonic acid; DANS-NH_2, dansyl amide; DANS-Hyp, dansyl hydroxyproline; ε-DANS-Lys, ε-dansyl lysine; bis-DANS-Lys, didansyl lysine; O-DANS-Tyr, O-dansyl tyrosine; etc.

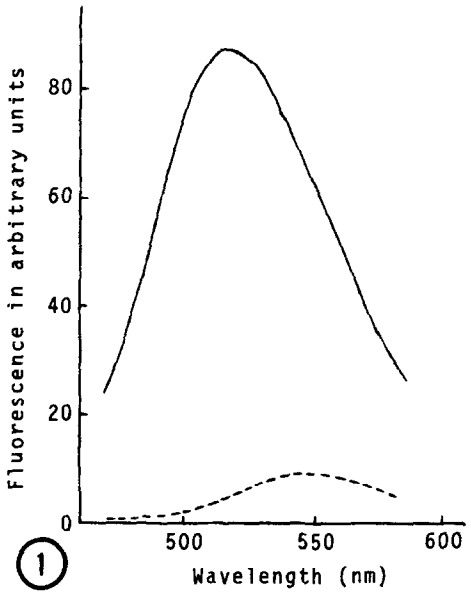
MATERIALS AND METHODS

Dansyl L-amino acids were purchased from Seikagaku Kogyo Co. Stock solutions of dansyl amino acids were prepared by firstly dissolving 5 to 10 mg of the samples in 2 ml of methanol and then diluting to 50 ml with redistilled water. The stock solutions were further diluted as indicated in each experimental regend. C7A was the gift of Mr. K. Tsukashima, Nikken Kagaku Kogyo Co., and once recrystallized from water before use.

For assay of dansyl amino acids, 2 ml of the sample solution was mixed with 2 ml of 16.0 mM C7A solution in 0.2 M phosphate buffer, pH 7.4 and the resultant fluorescence intensity was measured at 22.5°. For titration of dansyl amino acids with C7A, the C7A concentration was varied from 1.3 to 8.0 mM and the fluorescence intensity was evaluated at three different temperatures of 4.0, 22.5, and 40.0°. The measurement of fluorescence was performed with a Hitachi Model 103 fluorescence spectrophotometer, thermostated within an accuracy of 0.1°.

RESULTS AND DISCUSSION

Establishment of the assay: Addition of C7A to dansyl amino acid solutions brought shift of fluorescence spectra to shorter wavelengths with enhancement of fluorescence intensities in the range of 5 to 18 nm. Fig. 1 shows fluorescence spectra of dansyl valine in the presence and absence of 8.0 mM C7A. Fluorescence maxima were observed within the range of 510 to 525 nm. Fig. 2 indicates the fluorescence intensity at 515 nm of dansyl valine plotted against varied C7A concentrations. The intensity increased with the concentration of C7A and reached a plateau at 6.7 to 8.0 mM C7A. In the following experiments, The C7A concentration was fixed to an excess concentration of 8.0 mM unless otherwise indicated. Maximum intensity of the fluorescence was observed in a pH region of 6.0 to 10.0. The fluorescence was stable for two days at pH 7.4 at room temperature.



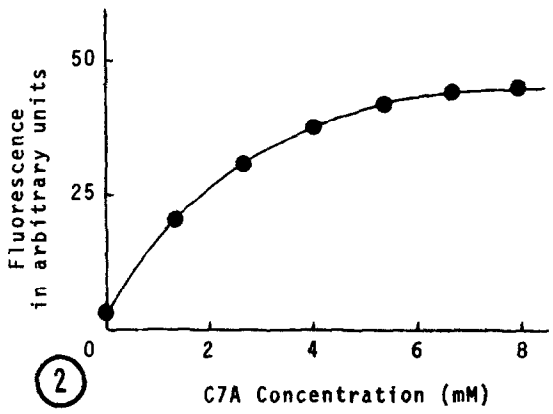


Fig. 1. Fluorescence spectra of 2.6×10^{-5} M DANS-Val in the presence (———) and absence (- - - - -) of 8.0×10^{-3} M C7A. Excitation: 365 nm.

Fig. 2. Fluorescence intensities of 1.3×10^{-5} M DANS-Val in the presence of varied C7A concentrations. Excitation: 365 nm. Emission: 515 nm.

Fig. 3 illustrates standard curves for determination of dansyl amino acids. Although fluorescence intensities were only about a half the intensities measured in methanol, dansyl amino acids were found to be determined up to 0.2 nanomoles because of the high reproducibility of the standard curves. This procedure may be followed more simply than that using organic medium for the fluorescence measurement.

Interferences of several compounds with fluorescence were examined by measuring fluorescence intensities in the presence of these compounds (Table I). Urea, NaCl, $NaHCO_3$, L-lysine and D-glucose had practically no effect on the intensity at a concentration level of 25 mM. Perchlorate and benzoate markedly quenched the fluorescence, which is presumably due to competition of these materials with dansyl amino acids for the binding site of C7A molecule. Cramer and co-workers (5) have demonstrated that perchlorate anion forms a complex with cyclohexaamylose.

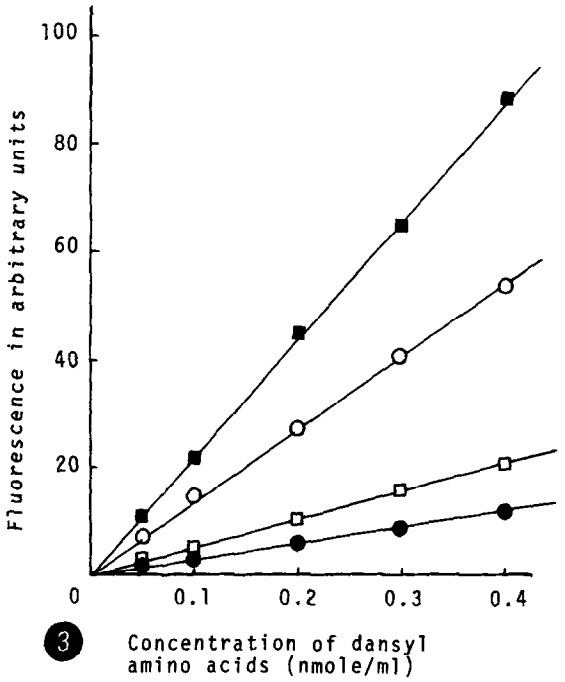
Table I. Relative fluorescence intensity of DANS-Phe and DANS-Val in the presence of several compounds in addition to 8.0 mM C7A.

	50.2 μM DANS-Phe	37.4 μM DANS-Val
None	100.0	100.0
+ Urea, 250 mM	100.0	99.5
NaCl, 250 mM	103.0	103.8
$NaHCO_3$, 25 mM	101.0	101.1
250 mM	107.3	108.0
L-Lysine, 250 mM	103.8	105.8
D-Glucose, 250 mM	105.0	104.6
$NaClO_4$, 25 mM	88.0	86.3
250 mM	46.5	45.0
Sodium Benzoate, 25 mM	83.3	81.0
250 mM	28.3	25.4

Occurence of perchlorate and benzoate at such a high level of concentration as used above may be less expected in biological samples.

Thermodynamic study: The interaction of C7A with dansyl amino acids were further studied thermodynamically, assuming that the enhancement of fluorescence is resultant from complex formation between them. Titration data of dansyl amino acids with C7A were analyzed by the use of Benesi and Hildebrand equation (6). A plot of $C_a/\Delta I$ against $1/C_d$ for dansyl valine was found to be straight lines and the association constant was temperature dependent (Fig. 4). C_a and C_d indicate the concentrations of dansyl amino acids and C7A, respectively. ΔI is the increment of fluorescence intensity of dansyl amino acids after addition of C7A.

Table II shows the summary of thermodynamic study. There was no remarkable difference among free energy changes, while the enthalpies



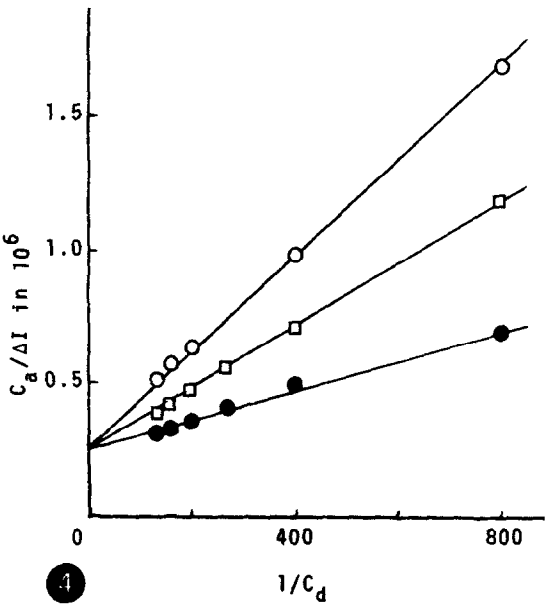


Fig. 3. Standard curves for dansyl amino acids: DANS-Leu (■), bis-DANS-His (○), O-DANS-Tyr (□), and DANS-Asp (●). Standard curves for DANS-Ile, DANS-Val, bis-DANS-Lys, DANS-Ala, ε-DANS-Lys, DANS-Phe, DANS-Thr, DANS-Glu, DANS-Arg, DANS-Pro, DANS-Asn, DANS-Gln, DANS-Hyp, DANS-Ser, DANS-Cys-SO_3H, and DANS-Trp lie between those for bis-DANS-His and O-DANS-Tyr.

Fig. 4. Determination of the association constants for the interaction of C7A with DANS-Val according to Benesi and Hildebrand at 40.0° (○), 22.5° (□), and 4.0° (●).

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and entropies varied with amino acids. The values of entropy changes for relatively hydrophobic dansyl amino acids such as dansyl phenylalanine, didansyl lysine or dansyl valine were negative or approximately zero. This may be explicable according to the concept by Bender and co-workers (7) that water molecules in the cavity of C7A cannot form their full complement of hydrogen bonds because of steric restrictions. Straub and Bender (8) observed negative entropy changes for the association of C7A with benzoyl acetic acid derivatives. On the other hand, relatively hydrophilic dansyl amino acids such as dansyl serine, dansyl glutamic acid or dansyl alanine showed positive entropy changes. This fact is hard to be interpreted by the above mentioned concept and may be explained as

Table II. Thermodynamic data in interaction of C7A with dansyl amino acids.

Dansyl amino acid	K x 10^{-2} ($mole^{-1}$) (22.5°)	ΔG ($kcal \cdot mole^{-1}$) (22.5°)	ΔH ($kcal \cdot mole^{-1}$)	ΔS ($kcal \cdot mole^{-1} \cdot deg^{-1}$)
DANS-Phe	0.9	-2.6	-5.6	-10.0
bis-DANS-Lys	1.9	-3.1	-4.2	-3.7
DANS-Asp	1.4	-2.9	-3.1	-0.7
DANS-Val	2.0	-3.0	-3.1	0.2
DANS-Trp	4.2	-3.5	-3.4	0.5
DANS-Hyp	1.3	-2.8	-2.4	1.5
DANS-Cys-SO_3H	1.5	-2.9	-2.4	1.9
DANS-Leu	2.0	-3.1	-3.7	2.0
ε-DANS-Lys	2.5	-3.2	-2.6	2.1
DANS-Ile	1.9	-3.1	-2.4	2.2
DANS-Arg	1.1	-2.7	-2.1	2.2
DANS-Pro	2.2	-3.2	-2.2	3.2
DANS-Thr	1.8	-3.0	-2.0	3.5
DANS-His	1.6	-3.0	-1.8	3.8
DANS-Gln	1.3	-2.9	-1.6	4.4
DANS-Asn	1.1	-2.8	-1.4	4.6
O-DANS-Tyr	1.1	-2.7	-1.3	4.8
DANS-Ala	1.9	-3.1	-1.7	5.4
DANS-Glu	1.0	-2.7	-1.0	6.7
DANS-ser	1.6	-3.0	-0.9	7.0
DANS-OH	1.4	-2.9	-0.8	7.2

described below. Hydrophilic amino acid moieties carry more water molecules hydrogen-bonded to its polar sites than hydrophobic ones, and release of the water molecules in the interaction results in increase of entropy.

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