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Note

Binding constants for complexes of α-cyclodextrin with L-phenylalanine and some related substrates

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Cyclodextrins are cyclic oligomers of six or more (1 → 4)-linked α-D-glucopyranose units. The six-, seven-, and eight-unit compounds are called cyclomaltohexaose (α-cyclodextrin), cyclomaltoheptaose (β-cyclodextrin), and cyclomalto-octaose (γ-cyclodextrin), respectively. These substances are produced by the action of *Bacillus macerans* amylase on starch. Because the cyclodextrins possess a cavity of molecular dimensions, they are able to act as "host" molecules for the formation of host–guest inclusion complexes. Many reviews have been published [1–10].

A study in one of our laboratories [11] recently showed that β-cyclodextrin significantly reduces the temperature-induced polymerization of β-casein. The mechanism of this inhibition is not known. Because the cyclodextrin–casein system is extremely complex, we have initiated studies on relatively simple model systems in order to learn something about possible sites and energetics of interaction. Inoue et al. [12] have reported that inclusion complex formation with phenylalanine dipeptides involves insertion of the aromatic side chain into the cyclodextrin cavity. Our systematic investigations into complex formation between α-cyclodextrin and many classes of aromatic substrates [13–17] therefore led us to design the present study of the complexing of α-cyclodextrin with phenylalanine and with some phenylalanine derivatives. At the time this work was begun we were aware of a literature report [18] on α-cyclodextrin–phenylalanine complexation, but the reported equilibrium constant seemed to us to be highly dubious. While our work was in progress other values were published [19,20], and these are in close agreement with our result, as will be described subsequently.

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EXPERIMENTAL

Materials.—α-Cyclodextrin (αCD), research grade, was obtained from American Maize Products Co., Hammond, IN and was used without further purification. The moisture content was determined by oven drying at 120° for 3 h and it was found that two moles of water were present per mole of αCD. The moisture content was taken into account when calculating the mass of αCD required in making solutions. L-Phenylalanine (purity 99% or greater) was from Sigma Chemical Co., St. Louis, MO. Three tripeptides, phenylalanylglycylglycine (Phe-Gly-Gly), glycylphenylalanylglycine (Gly-Phe-Gly), and glycylglycylphenylalanine (Gly-Gly-Phe), all research grade, were obtained from Serva Biochemicals, Westbury, NY. The degree of purity was 98% or greater for all tripeptides and they were used without further purification. The tripeptides were stored in sealed containers at 4° until needed. Phenylalanine methyl ester hydrochloride was obtained from Aldrich Chemical Co., Milwaukee, WI (degree of purity 99% or greater). Tris(hydroxymethyl)aminomethane (TRIS) and tris(hydroxymethyl)aminomethane hydrochloride, both research grade, were from Sigma Chemical Co., St. Louis, MO. Sodium chloride (analytical reagent grade) was from Mallinckrodt Chemical Works, Paris, KY. Durapore membrane filters, 0.22-μm pore size, were from Millipore Corporation, Bedford, MA. All solutions were prepared fresh at the start of each experiment, using doubly distilled water.

Apparatus.—Spectrophotometric measurements were made using a Cary 1 UV-VIS spectrophotometer from Varian Instruments, Sugar Land, TX. The spectrophotometer was fitted with jacketed cell compartments for temperature control at 25.0 ± 0.2°. Measurements of pH were made with a Fisher Accumet pH meter, Model 815 MP (Pittsburg, PA), in conjunction with a Corning semimicro combination electrode (cat. No. 476541; Park Ridge, IL).

Procedures.—The buffer was pH 9.00 TRIS, with the ionic strength adjusted to 0.10 M with NaCl; the buffer solution was filtered through a membrane filter. All measurements were made at 25°. The buffer pH was selected to ensure that αCD is not appreciably ionized ($pK_a = 12.3$) [21]. TRIS buffer does not interfere with the measurement of αCD binding constants [22].

A solution of 0.1 M αCD was prepared in 0.1 M TRIS buffer. Solubilization of αCD was achieved by stirring at moderate speed for 30 min at room temperature. Five different substrate stock solutions were used: 0.049 M L-phenylalanine, 0.054 M solutions of each of the three tripeptides (Phe–Gly–Gly, Gly–Phe–Gly, and Gly–Gly–Phe), and 0.060 M L-phenylalanine methyl ester · HCl. They were prepared by dissolving accurately weighed amounts of the solutes in the TRIS buffer. For each substrate, the following procedure was then used.

To prepare a series of sample solutions with increasing αCD concentration and constant substrate concentration, increasing volumes of 0.1 M αCD solution (from 1.0 to 9.0 mL) were pipetted into 10 mL volumetric flasks. Next, 1.0 mL of the substrate stock solution was added to each flask, and TRIS buffer was added to

bring the contents to volume. A flask containing an identical amount of substrate but no CD was also prepared; this was used for obtaining the absorbances of the substrate in the absence of αCD. Reference solutions were prepared for each sample solution, having identical αCD concentrations in TRIS buffer but no substrate. All flasks were placed in a water bath at 25 ± 2° for at least 30 min to assure temperature equilibration.

Absorption spectra of each substrate in the presence and absence of αCD were obtained to permit selection of wavelengths at which the substrate absorption changes substantially upon complexation. All substrates showed similar absorption spectra (1-cm quartz cuvettes), and the two wavelengths selected were 251.5 and 257.0 nm. The baseline for each absorbance reading was set at that of the corresponding reference solution, i.e., the solution with an identical CD concentration but no substrate. The temperatures of the solutions in the cuvettes were measured with thermocouples before recording each absorption spectrum. For each substrate, replicate experiments were conducted on different days to provide information on the precision of the data.

Data analysis.—Since the spectrophotometric procedure for the measurement of complex binding constants is well known, a brief summary will suffice [23]. The binding isotherm is

$$\frac{\Delta A}{b} = \frac{S_t K_{11}\, \Delta\epsilon_{11}[CD]}{1 + K_{11}[CD]}, \qquad (1)$$

where ΔA is the difference in absorbance, at a fixed wavelength, between sample solution (containing total substrate concentration S_t) and reference solution (without substrate), both solutions having the same total αCD concentration, $[CD]_t$. K_{11} is the binding constant for 1:1 stoichiometric complex formation, and $\Delta\epsilon_{11}$ is the difference in molar absorptivities of the complexed and uncomplexed substrate. Eq *2* is one of several linearized forms of eq *1*.

$$\frac{b[CD]}{\Delta A} = \frac{[CD]}{S_t\, \Delta\epsilon_{11}} + \frac{1}{S_t K_{11}\, \Delta\epsilon_{11}} \qquad (2)$$

It is particularly useful in the present instance because the experimental αCD concentrations are equally spaced, and their spacing is preserved in this plotting form.

In eqs *1* and *2*, $[CD]$ is the free (unbound) concentration of αCD, which is not known. The procedure is to make the initial approximation $[CD] = [CD]_t$, to make the plot of $[CD]_t/\Delta A$ vs. $[CD]_t$ according to eq *2*, and from the slope and intercept to obtain initial estimates of K_{11} and $\Delta\epsilon_{11}$. Then this provisional value of K_{11} is used in eq *3* (which is obtained from the mass balance for CD) to calculate the value of $[CD]$ corresponding to each $[CD]_t$.

$$[CD]_t = [CD] + \frac{S_t K_{11}[CD]}{1 + K_{11}[CD]} \qquad (3)$$

Now these values of $[CD]$, together with the estimates of K_{11} and $\Delta\epsilon_{11}$, are used in a nonlinear regression according to eq *1* to obtain improved estimates of K_{11} and $\Delta\epsilon_{11}$. Iteration between eqs *1* and *3* continues until the parameter estimates converge.

The nonlinear least-squares regression analysis was done with the SAS statistics program (SAS Institute, Inc., Cary, NC). Convergence of the parameter estimates is achieved when this criterion is met:

$$\frac{(SSE_i - SSE_{i-1})}{(SSE_i + 10^{-6})} < 10^{-8}$$

where *SSE* is the sum of standard errors.

RESULTS AND DISCUSSION

All of the substrates exhibited bathochromic (red) shifts in the presence of αCD. Fig. 1 shows the absorption spectra of Gly-Gly-Phe in the presence and absence of αCD. If only 1:1 stoichiometry is involved, the binding constant estimated according to eq *1* should be independent of wavelength, so two wavelengths were selected for binding-constant measurements. These wavelengths, 251.5 and 257 nm, which were chosen to maximize the absorbance differences, yield negative values of ΔA and $\Delta\epsilon_{11}$ (see Fig. 1).

It is evident from Fig. 1 that the experimental problem is difficult, because the spectral shift is small. Consequently we expect relatively imprecise parameter estimates. Fig. 2 shows plots according to eq *2* for Gly-Gly-Phe, and Fig. 3 is a plot of eq *1* for this substrate. These results are typical. The peptide Gly-Phe-Gly, however, exhibited anomalous behavior, in that it showed small spectral shifts that

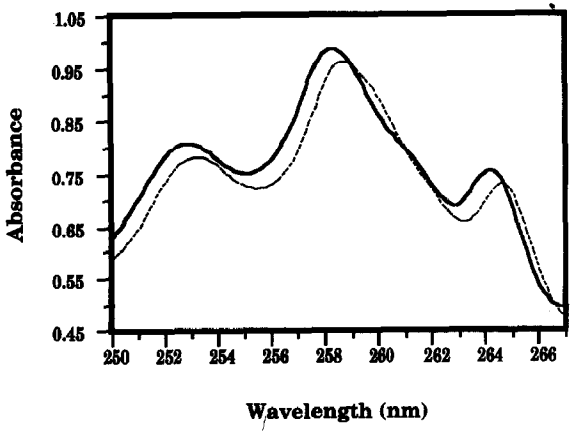

Fig. 1. Absorption spectra of 0.00538 M Gly-Gly-Phe in pH 9.0 TRIS buffer. Solid line, no αCD present; dashed line, with 0.09 M αCD.

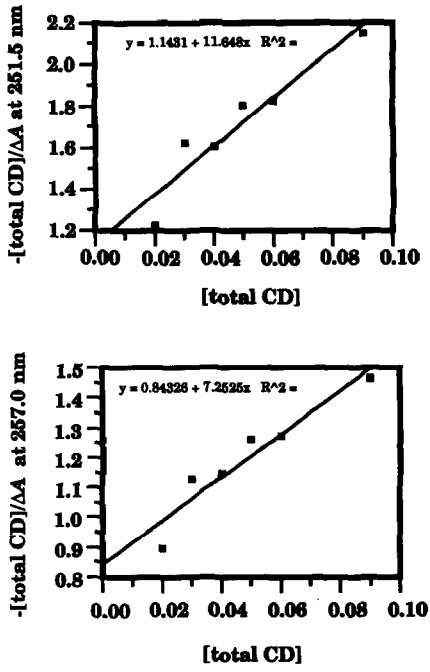

Fig. 2. Plots according to eq 2 for the Gly-Gly-Phe–αCD system at the designated wavelengths; conditions as in Fig. 1.

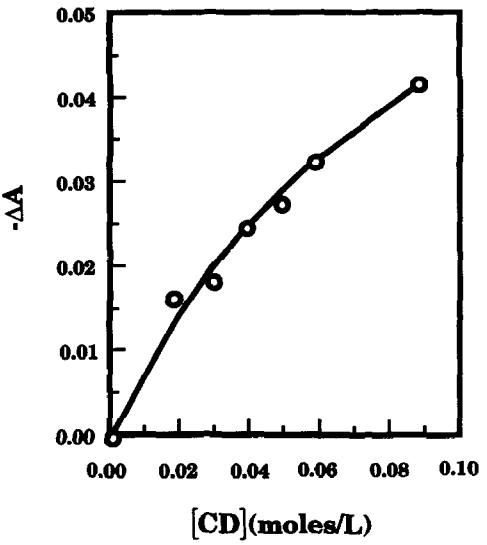

Fig. 3. Plot of ΔA (at 251.5 nm) against free αCD concentration for Gly-Gly-Phe. The points are experimental and the line is the final least-squares nonlinear regression line.

TABLE I

Estimated values of parameters for α-cyclodextrin-complex formation at pH 9.0 and 25°

Substrate	No. of determinations	K_{11} (M^{-1}) [a]		$\Delta\epsilon_{11}$ (M^{-1} cm^{-1}) [a]	
		251.5 nm	257 nm	251.5 nm	257 nm
L-Phenylalanine	3	12.4 (2.9)	11.6 (3.1)	−19.7 (3.5)	−29.7 (2.9)
Phenylalanine methyl ester	2	19.2 (2.6)	18.4 (5.3)	−14.3 (0.0)	−21.6 (2.3)
Phe–Gly–Gly	3	11.7 (2.1)	16.4 (7.5)	−16.2 (1.7)	−20.8 (1.5)
Gly–Gly–Phe	3	10.4 (1.2)	8.1 (1.1)	−19.8 (5.3)	−32.2 (8.4)

[a] The quantities in parentheses are standard deviations. Within-run standard errors in K_{11} ranged from 1.6 to 5.5.

were not smooth functions of αCD concentration. This problem was not resolved, and it therefore was not possible to estimate a binding constant for this peptide.

Table I lists the means of the K_{11} and $\Delta\epsilon_{11}$ values obtained in this work. The essential conclusions are that K_{11} is modest in magnitude, that it apparently does not depend on wavelength, and that it is not greatly dependent upon the substrate structure for this related set of substrates. At pH 9 phenylalanine and the peptides are negatively charged, whereas the methyl ester of phenylalanine is neutral. The value of K_{11} for the methyl ester does appear to be significantly larger than the values for the other substrates; this is consistent with the view that high polarity is not conducive to strong binding in the relatively nonpolar CD cavity. On the basis of much prior work with CD complexes, it is most reasonable to expect the aromatic ring to be the binding site. This is consistent with the observed proton NMR chemical shifts [24], and with ^{13}C-NMR correlation-time measurements [5].

The magnitudes of these K_{11} values are comparable to those of other amino acids having nonpolar side chains [25]. Paduano and coworkers have measured K_{11} for the αCD–L-phenylalanine system by calorimetry [19] and by a diffusion technique [20], obtaining values of 13.6 M^{-1} and 9.7 M^{-1}, respectively. These results are in good agreement with our mean value (Table I) of 12 M^{-1}. We believe that the earlier calorimetric result of Lewis and Hansen [18] can be rejected.

We can also examine the consistency of our αCD–phenylalanine result with published data on related substrates, namely those in which binding takes place at the aromatic ring in a monosubstituted benzene. This comparison requires microscopic (site) binding constants at the aromatic ring, for this is the most reasonable assignment of K_{11} for phenylalanine. Four such examples are known to us: these are benzoate anion (K_{11} = 11.5 M^{-1}) [22], phenolate (K_{11} = 10.9 M^{-1}) [15], aniline (K_{11} = 8.8 M^{-1}) [14], and biphenyl (K_{11} = 50 M^{-1}, or a site binding constant of 25 M^{-1}) [17]. The result for L-phenylalanine (12 M^{-1}) is fully consistent with these observations, and the very similar K_{11} values found for the other substrates in the present study suggest that the aromatic ring is the common binding site.

We have assumed a 1:1 stoichiometric relationship in this study. The small spectral shifts and modest binding constants do not allow this assumption to be

rigorously tested, but several lines of evidence are consistent with it. First, the 1:1 binding isotherm, eq *1*, satisfactorily accounts for the data; second, K_{11} appears to be independent of wavelength; and third, different experimental methods (spectrophotometry, calorimetry, and diffusion) yield essentially the same experimental K_{11} value [26]. With eq *4*, the 1:1 binding isotherm [27],

$$f_{11} = \frac{K_{11}[L]}{1 + K_{11}[L]} \qquad (4)$$

we can calculate the extent of binding at any reasonable ligand concentration. In eq *4*, f_{11} is the fraction of substrate bound, and $[L]$ is the free ligand concentration (CD in our case). For example, with $K_{11} = 12\ M^{-1}$ and $[L] = 0.09$, $f_{11} = 0.52$. This is sufficient extent of binding to provide a reasonable estimate of the binding constant, though it does not permit the assumption of 1:1 stoichiometry to be carefully examined.

The essential finding of this work is that the binding of αCD with phenylalanine and phenylalanine derivatives takes place to a modest extent, an extent that is consistent with observations on other related small molecules. Binding probably occurs at the aromatic ring.

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