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Association Constant of the Sex-Specific Agglutinin in the Yeast, *Hansenula wingei**

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ABSTRACT: Association constants were determined for absorption of the sex-specific agglutinin from mating type 5 of *Hansenula wingei* by cells of type 21, the opposite mating type. Absorption was determined by following the radiolabeled sulfur in purified agglutinin extracted from cells grown in ^{35}S-labeled medium. Association was proved to be reversible under conditions where cell agglutination was weak, and was inferred to be reversible and faster than agglutination under nearly all conditions. The reactions could be characterized by a single association constant dependent on pH. The standard free energy of association was maximum at pH 4 and was approximately −14.5 kcal/mole of 5-agglutinin particles. This high value indicates cooperation among several binding sites may occur.

In sexual agglutination, cells of the two mating types of a species of yeast agglutinate on mixing. Some pairs do so vigorously (Wickerham, 1956). In *Hansenula wingei* NRRL-2340, a species which may have strongly agglutinative mating types, the agglutinative factor from mating type 5 can be digested from the cells with proteases such as subtilisin. This factor, which we named 5-Ag[1] for convenience, agglutinates type 21 cells, the opposite mating type, and is strongly absorbed by them. The agglutination and absorption are specific for type 21 cells of *H. wingei*.

In this report the absorption isotherm of 5-Ag on type 21 cells are examined under various conditions to determine the association constants and free energy of association. While this information is interesting in itself, our primary purpose is to use these features of the binding reaction in future work to examine further the nature of 5-Ag binding. The 5-Ag particles are macromolecules which appear to have about six active sites for binding (Taylor and Orton, 1968). The particles have a molecular weight of about 10^6 and are composed of 4% protein and the remainder, mannan. The active sites depend on the integrity of the disulfide bonds associated with them, of which there is one per site. It is possible that several of these active sites act cooperatively. In a report to be submitted, we examine the interrelation of these active sites, using the procedures developed here.

Methods

Most of the procedures have been described (Taylor and Orton, 1967, 1968). Absorption of 5-Ag by type 21 cells was determined by following ^{35}S label. Aliquots of 5-Ag were mixed both with type 5 cells and type 21 cells, at identical concentrations, to make 6 ml of reaction mixtures. After incubation with stirring for 30 min, the cells were centrifuged off, and duplicate 2-ml portions were taken from the supernatant solutions for scintillation counting. The 30-min reaction times were determined to be adequate by experiment. When reaction mixtures were incubated for times from 10 to 60 min, all results were identical. To allow for possible variation in reaction rate the 30-min time was chosen.

The counts from the supernatant solution from type 21 cells corresponded to the free 5-Ag; those from the 5 cells, to the original input. The concentration of absorbed 5-Ag is found by difference. Six to ten sets of reaction mixtures containing different doses of 5-Ag were made having the same concentration of cells. Controls with no cells gave counts within a few per cent of the supernatant solutions from type 5 cells. Suitable blanks and ^{35}S standards were counted also. Counts were done in a Beckman[2] LS 100 scintillation counter with optimum E^2/B for ^{35}S; each vial was counted twice for 50 min or 3% (2σ). For a reasonably precise experiment, it was important that the doses of 5-Ag and the cell concentration were nearly optimum values.

Early work showed that at low pH, 5-Ag had some nonspecific absorption to yeast cells. This was attributed to the presence of phosphate groups in 5-Ag. Most of the 5-Ag in this unit was from cells grown in a low phosphate medium, for which the nonspecific absorption was minimized. At pH higher than about 3.5, no difference in absorption by the two types of 5-Ag was noted.

The growth medium contained, per liter, 3 g of $NaNO_3$, 0.1 g of KH_2PO_4, 0.3 g of $MgCl_2 \cdot 6H_2O$, 20 g of glucose, 25 mg of $MgSO_4 \cdot 7H_2O$ labeled with $[^{35}S]H_2SO_4$, trace elements, and vitamins. The higher phosphate medium used previously (Taylor and Orton, 1968) contained 1 g of KH_2PO_4/l. The formula given there is partly in error.

A digital computer was used for some calculations concerning absorption isotherms, numerical analysis, and mathematical models.

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[1] Abbreviations used are: 5-Ag, 5-agglutinin; 12.5 × limit, the absorption limit of type 21 cells calculated by proportion to 12.5 × cell concentration.

[2] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

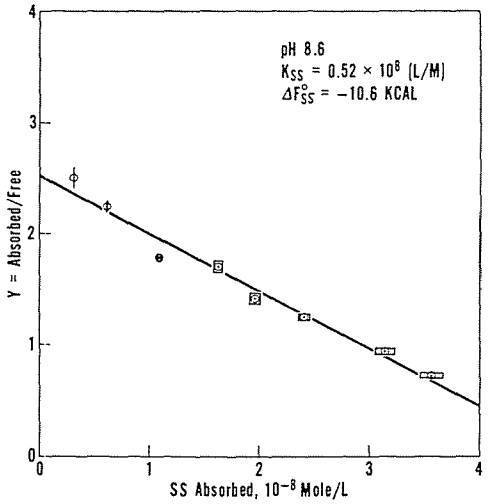

FIGURE 1: Absorption isotherm of 5-Ag on cells of *Hansenula wingei* type 21, at pH 8.6, 25°. Cell concentration = 12.5×. SS absorbed indicates moles of disulfide absorbed.

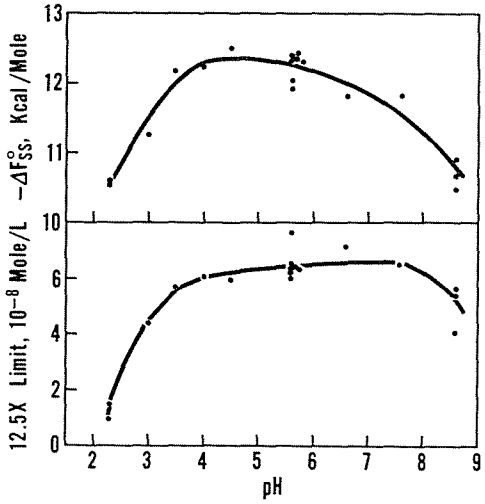

FIGURE 3: Effect of pH on binding energy, $-\Delta F_{ss}^{\circ}$, and on cell absorption limit at 12.5×. Cells are from lot W.

Results

Typical absorption isotherms are shown in Figures 1 and 2, which are like Scatchard (1949) plots except that both bound and free sulfur concentrations are given in units of moles of disulfide per liter. The ordinates, Y, are bound concentration divided by free concentration at equilibrium.

The slopes of the lines give the apparent association constants per mole of disulfide, K_{ss} (liters/mole), when concentrations on the abscissa are given as moles of disulfide groups per liter

$$K_{ss} = \Delta Y/\Delta(L - X) \quad (1)$$

where X is the concentration of disulfide absorbed by the cells, and L is the cell absorption limit at $Y = 0$. The apparent standard free energy of association, ΔF_{ss}°, is calculated by

$$\Delta F_{ss}^{\circ} = -RT \ln K_{ss} \quad (2)$$

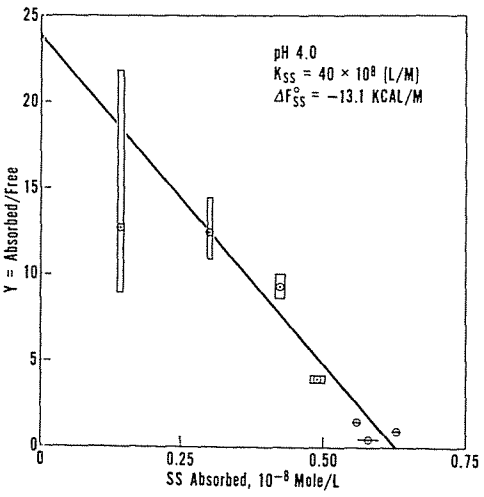

FIGURE 2: Absorption isotherm of 5-Ag on type 21 cells at pH 4.0, 25°. Cell concentration = 3×.

where R is the molar gas constant and T the absolute temperature.

The data are averages of duplicate samples, and the surrounding boxes indicate approximately the 34% probability (one standard deviation when distribution is normal) of position of the mean value. When the association constant was fairly low, as in Figure 1, a regression line could be fit with good precision. When the association was strong, as in Figure 2, the amount of free 5-Ag was often too small to give precise data at low additions.

Figures 1 and 2 show the range of precision observed. The slopes could be found within a factor of two at worst, which corresponds to a difference of 0.4 kcal/mole in free energy. This precision is quite adequate for present purposes as it is only a small percentage of the high values found.

That pH of the medium affects free energy is shown in Figure 3. The free energy is most negative in the range of pH 3.5 to 6.5, similar to the range for sexual agglutination in *H. wingei* (Taylor, 1964).

Extrapolation of the lines in Figures 1 and 2 to $Y = 0$ gives the cell absorption limit. The values are different in the two figures because cell concentration was set at optimum values for the isotherms. The limits converted by proportion to those for cells at 12.5× standard concentration (12.5× limit) are shown in Figure 3 to be approximately constant over the pH range 3.5 to 7.5.

Variation Among Lots of Cells. Several different 5-Ag preparations were used in the pH dependence study illustrated in Figure 3. While there was little difference in properties among different lots of 5-Ag, the cells did vary considerably. In Table I are the results of a comparison of three separate stocks of cells and two lots of 5-Ag. In particular, cell absorption limits are variable among lots of cells, whereas free energies vary only slightly more than our accepted experimental error. Owing to these variable absorption limits, comparison studies can be made with only one stock of cells. The values found for free energy can be compared among cell lots.

Purity of 5-Ag. Because values of Y are quite high in some experiments, and because a high ratio, Y, tends to emphasize

TABLE I: Comparison of 5-Ag Preparations and Type 21 Cell Stocks in Absorption Isotherms at pH 5.6.

Type 21 Cell Stock	12.5× Limit (10^{-8} mole of SS/liter)		$-\Delta F_{ss}°$ (kcal/mole of SS)	
	5-Ag A	5-Ag B	5-Ag A	5-Ag B
W	6.4	6.4	12.3	12.4
X	8.1	8.8	12.5	12.6
Y	3.3	3.2	12.8	12.8

the effect of inert contaminants in the isotherm, it is important that 5-Ag be sufficiently free from contaminants. In Table II are given the observed parameters of absorption isotherms at different concentrations of cells. The cell concentration is that relative to our standard concentration (about 50×10^6 cells/ml). The 12.5× limit is nearly constant. Y_0 is the limiting absorption ratio at $X = 0$ calculated from the regression line.

The effect of a fraction, α, of inactive material in 5-Ag on the absorption isotherm is shown by the theoretical curves drawn in Figure 4, based on the equation:

$$Y = \frac{1 - \alpha}{\frac{1}{K_{ss}(L - X)} + \alpha} \quad (3)$$

where α is the inactive fraction. For the active material alone, $K_{ss}L$ was set at 80, and the isotherm is linear. As the contaminant fraction is increased, the curves become concave downward and fall lower in the field. For $K_{ss}L = 80$, even 1% contaminants have a pronounced effect. As $K_{ss}L$ increases, the effect becomes larger. This effect is much more pronounced here than in the usual equilibrium dialysis test, where the amount absorbed is only a relatively small fraction of the total quantity available.

In a series of isotherms at different cell concentrations, then, even if the curvature cannot be detected, the apparent association constant should decrease with concentration and the apparent 12.5× cell limit should increase. The values of α in Table II were calculated by numerical analysis to minimize the sum of squares of the derivatives of the Y values of the data from the curve of eq 3. The values used for K_{ss}, 140×10^8, and L at 12.5× cell concentration, 2.6×10^{-8}, were obtained by extrapolating values of K_{ss} and 12.5× limit in Table II to zero cell concentration.

The values of α (Table II) are small. Variation of the association constants with cell concentration can be ascribed to a small fraction, less than 1%, of sulfur from inactive material in 5-Ag. This purity is quite adequate for these experiments, where the ratios, Y, are less than about 50, as in most of our work.

Reversibility. Reversibility of the absorption reaction was tested by observing the effect of diluting a concentrated absorption mixture. Some of the absorbed 5-Ag should be released on dilution, thus increasing the final free 5-Ag concentration above that predicted for dilution of free 5-Ag only. For each experiment, three absorption isotherms

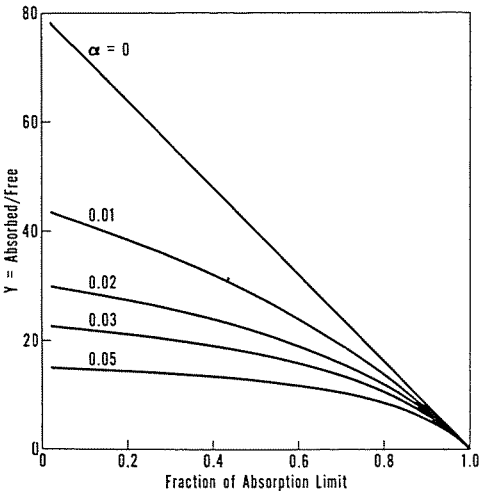

FIGURE 4: Theoretical absorption isotherms for mixtures of active 5-Ag and a fraction, α, of inactive contaminant. $K_{ss}L$ was set at 80.

of several 5-Ag doses were run: (1) an absorption run incubated with concentrated mixtures, (2) a partially reversed isotherm with reaction mixtures identical with (1) and subsequently diluted and reincubated, and (3) an absorption run with reaction mixtures equivalent to the final dilute ones in (2).

Results are given in Table III. Reversal is the 5-Ag released on dilution calculated from isotherms 1 and 2, divided by that expected from 1 and 3. Displacement is the 5-Ag released on dilution divided by total 5-Ag in the reaction mixture. Both are average values of the several doses.

At pH 4, where agglutination and absorption are strongest, reversibility appears poor. However, it is probable that agglutination interferes with the desorption experiments. Absorption occurs very rapidly, but agglutination usually occurs much slower (data not given). This relationship is the usual one in systems of particles flocculated by polymers (Healy, 1961; Peterson and Kwei, 1961; Black *et al.*, 1966) and for antibody reactions (reviewed by Nisonoff and Thorbecke, 1964). Then absorption equilibrium should be reached or approached before agglutination occurs. Because on dilution the agglutinated cell mass could either prevent or

TABLE II: Effect of Cell Concentration on the Absorption Isotherm of 5-Ag at pH 4.

Cell Concentration	12.5× Limit (10^{-8} mole of SS/liter)	Y_0	K_{ss} (10^8 liter/mole)	$-\Delta F_{ss}°$ (kcal/mole)	α
1	2.63	22	103.4	13.71	0.015
3	2.64	88	139.2	13.88	0.000
12.5	2.80	360	128.4	13.74	0.000
62.5	(2.63)[a]	205	14.6	12.46	0.004

[a] This value was assumed. Because agglutination was heavy at larger doses of 5-Ag, the limit could not be found.

TABLE III: Reversibility of Absorption of 5-Ab on Type 21 Cells.

pH	Reversal[a]	Displacement[b]
2.65	0.98	0.14
4.0	0.5	0.015
4.0 to 2.4[c]	0.97	0.7
4.0 (reduced)[d]	0.99	0.27

[a] Reversal is the observed dissociation on dilution, given as fraction of expected reaction. [b] Displacement is the observed dissociation on dilution, given as fraction of total 5-Ag in the system. [c] Diluted from pH 4 to a final pH 2.4. [d] This 5-Ag preparation was partially inactivated by treatment with mercaptoethanol (see text).

retard the shift to a new desorption equilibrium on the cell surfaces, we believe that the absorption isotherms give more nearly the correct result. At very high concentrations of both cells and 5-Ag, the cells quickly agglutinate and may interfere even with absorption equilibrium, as indicated in Table II for 62.5× cell concentration. Otherwise, the general agreement of experiments in Table III and elsewhere suggests indirectly that equilibrium was reached during absorption.

In the other experiments in Table III the cells were not agglutinated during desorption, reversibility was satisfactory, and the displacement was sufficiently high for a reasonable test. The reduced 5-Ag, which is absorbed but is not highly agglutinative, was also satisfactorily reversible. This 5-Ag had 42% intact disulfide bonds, the C component was removed on G-100 Sephadex (Taylor and Orton, 1968), and the apparent standard free energy of association was lower, about −10 kcal/mole.

Discussion

The results consistently show that absorption is characterized by a single association constant dependent on pH. The slight variation with concentration of cells can be ascribed to a very low level of contaminating ^{35}S label, or inactive 5-Ag. While different lots of cells were variable, the apparent standard free energy of association was quite uniform at a single pH.

While all the absorption isotherms appeared to be linear, the association constants and corresponding free energies are probably average values, perhaps only tenuously associated with an intrinsic binding constant. This point will be developed in further reports.

Absorption equilibrium was proved to be reversible if the cells were not agglutinated. Allowing for relative rates of absorption compared to agglutination, we assume the absorption data indicate true equilibrium under all conditions. However, even if the worst conditions at pH 4 should prevail, an error of even 50% in free concentration would contribute a relatively small error to the free energy. For future work, it is noted that partially inactivated 5-Ag is absorbed reversibly, and therefore the association of such preparations can be interpreted unequivocally in thermodynamic terms.

Standard Free Energy of Association. The association constants above were given on the basis of molar concentration of disulfide bonds. Since we reported (Taylor and Orton, 1968) that the average 5-Ag particle contains about six disulfide bonds each, a correction can be applied to convert to the usual units of molar concentration of absorbed particles.

Our present lots of 5-Ag appear to be larger in molecular weight. The sedimentation coefficients were about 20% higher than previously reported (Taylor and Orton, 1968). The molecular weight should then be about 40% higher, and the sulfur content should be 14 per particle instead of 10. Sulfur content of the purified 5-Ag was the same as before, about 10 moles/940,000 g. If we assume there are six disulfide bonds per particle, then the association constants are corrected by dividing by six. The standard free energy of association of 5-Ag is found by adding $-RT$ ln 6 or −1.07 kcal/mole ΔF_{ss}° in eq 1, and the free energy of 5-Ag at pH 4 calculated from the data of Figure 2 should be $\Delta F_p^{\circ} = -13.1 - 1.07 = -14.2$ kcal/mole particles. The average value of several experiments is −14.5 kcal/mole, although it does depend somewhat on the cells used for absorption. The uncertainty over the molecular weight is only minor, since assignment of either six or of seven disulfide bonds per particle changes the derived binding energy by only 0.1 kcal.

The free energy value of −14.5 kcal/mole is high for a reversible biological reaction. Antibody–hapten binding free energies as high as this one have been observed only a few times (Karush, 1962). Evidently, either the complementary binding sites interact strongly or some cooperative effect occurs among binding sites on one particle.

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