the same binding titer for piericidin in intact, lyophilized, and reconstituted samples. While it is not likely that the added CoQ_{10} molecules which function in the regenerated system may be bound differently than in intact preparations (Ernster *et al.*, 1969a,b), the binding site titer for piericidin does not change during the entire cycle of treatments; so the same K_i value might apply to the untreated and reconstructed systems.

Acknowledgment

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Cooperation among the Active Binding Sites in the Sex-Specific Agglutinin from the Yeast, *Hansenula wingei**

Neil W. Taylor† and William L. Orton

ABSTRACT: Changes in properties of 5-agglutinin were examined during the course of the reaction to inactivate it by cleaving disulfide bonds. During this reaction both agglutinative titer and apparent free energy of absorption decreased rapidly with a relatively small proportion of disulfide bonds reacted. Fractionation experiments disclosed that very little biologically inactive material appeared until about 30% of the disulfide bonds were reacted. The 1.7S component which is released from 5-agglutinin during reaction retains the specific absorptive activity, though only very weakly. It is suggested that the high magnitude of the apparent free energy of association for 5-agglutinin, −14 kcal/mole, is the result of additive effect of several of the individual binding sites, each of which has a binding energy in the range −5 to −9 kcal per mole. A mathematical model based on these assumptions reproduces the behavior satisfactorily.

The sex-specific agglutinin derived from mating type 5 of the yeast *Hansenula wingei* agglutinates cells of type 21, the opposite mating type, and binds to them. Under favorable conditions the $\Delta F_p°$[1] is about −14 kcal/mole (Taylor and Orton, 1970). The 5-Ag has about six small 1.7S fragments which can be released by disulfide cleaving agents. These 1.7S fragments are associated with the biological activity. Presumably, 5-Ag has about six equivalent active sites which all contribute to its agglutinative and absorptive properties. This multiplicity of binding sites is somewhat more complex than occurs in a divalent antibody, and the resulting complexity seems to impart some unique properties to the 5-Ag particles.

Compared to particulate systems flocculated by polymeric materials, the small number of discrete binding sites on

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[1] Abbreviations used are: 5-Ag, 5-agglutinin, the sex-specific agglutinin from mating type 5 of *Hansenula wingei*; $\Delta F_p°$, apparent standard free energy of association for 5-Ag, in kilocalories per mole of particles.

5-Ag may be simpler and allow a simpler analysis of binding and flocculation than is possible in the polymer–particle system. The 5-Ag system seems nearly reversible, for example.

The 1.7S fragments are each joined to the parent 5-Ag by the one disulfide bond. Cleaving these bonds releases the 1.7S fragment and destroys the specific agglutinative activity. By progressive cleavage of the disulfide bonds, and examination of the biological activities and physical properties of the modified 5-Ag, we expect to learn something of the relationships among the active sites.

The results of these experiments show that the 5-Ag can be interpreted as a simple mass-action cooperation among the several active sites. No special structures or strong binding are required, and some of the unusual properties of 5-Ag are explained by this viewpoint.

Methods

Most of the procedures have been described (Taylor and Orton, 1967, 1968, 1970).

Association constants were determined as before (Taylor and Orton, 1970) with mating type 21 cell concentration varied from 3× to 25× standard concentration as necessary. Since absorption limits could not be determined when the association constants were very low, these constants were determined using the same limit as for intact 5-Ag.

Component A is the major component after disulfide reaction (see below). To determine the fraction of inactive material in component A of partially reacted 5-Ag, the preparation was fed onto a small column (1 × 6 in.) of type 21 cells in a cellulose powder matrix. Care was taken not to exceed the absorption limit of the column. The preparation was followed by pH 4 buffer to wash through unabsorbed material, and then the absorbed material was eluted with pH 1.7 buffer. The buffers contained 0.03% of phosphomannan NRRL Y-2448, needed to prevent nonspecific absorption in the column. About 85–95% of input was recovered in the column effluents. For preparation J discussed below, in which about eight times as much 5-Ag was reacted, our larger column (3 × 6 in.) of type 21 cells for 5-Ag purification was used.

Results

Changing Properties During Reduction. The disulfide groups of 5-Ag were cleaved with 2-mercaptoethanol. At pH 6.7 the reaction can be run at a reasonable rate and stopped after partial reaction by addition of a slight excess of *N*-ethylmaleimide (Gregory, 1955; Brewer and Riehm, 1967). A series of reactions was run for differing reaction times with ^{35}S-labeled 5-Ag. The partially reacted 5-Ag was then fractionated on Sephadex[2] G-100 (Figure 1). Component A, the major one in the ultracentrifuge pattern, and C, the 1.7S fragment, were recovered, which together contained most of the labeled sulfur. Since each 1.7S fragment contains one sulfur of its original disulfide bond, recovery of 38% of the label in component C implies that (100 − (2 × 38)) or 24% of the disulfide bonds remain intact. Recovery of ^{35}S label after fractionation was about 90% of input.

The general features of the disulfide cleaving reaction can be seen in Figure 2. The intact disulfide content decreased by an apparent first-order process. After about 40 min, only a few per cent of intact disulfide remained and the deviation from linearity is too near the experimental error involved to indicate significant difference from the first-order process.

Agglutinative titer decreased more rapidly with reaction time. It was determined in the mixture immediately after reaction.

The apparent association constants of component A on type 21 cells (Taylor and Orton, 1970) were determined. As shown in Figure 2 the constants, K_{ss} (calculated on the basis of moles of disulfide), decreased precipitously with reaction time.

Inactive Fraction. An important question at this point is whether a fraction of inactive material in component A was distorting the absorption isotherms. Even a small fraction of inactive material would reduce the apparent association constant because of the high values of the ratio, Y, achieved by unreacted 5-Ag (Taylor and Orton, 1970).

To probe this question the absorption isotherms were examined for evidence of inactive material. Figure 3 shows the absorption isotherm for a preparation of component A from 5-Ag reacted for 15 min.

The straight line is the linear regression line through the previously determined absorption limit for 5-Ag (corrected for sulfur loss). The dashed lines are curves describing theoretical behavior of two components in the reacted 5-Ag—an active fraction with the association constant indicated and an inactive fraction, α, constituting the remainder. The curves are least-squares regressions of Y on the data, optimizing for the parameter, α. The curve for $K_p = 330 \times 10^8$ indicates the expected result for unreacted 5-Ag and a fraction of inactive material. Obviously, the presence of inactive material cannot be the cause of the observed low association constants.

The amount of inactive fraction present was determined by passing recovered component A through a column of type 21 cells to absorb the active component. The ^{35}S label which flowed through the column without absorption presumably was an inactive 5-Ag. Figure 4 contains the results of several such experiments.

According to the data, no appreciable fraction of inactive material appeared until approximately 30% of the disulfide bonds had been cleaved. After about 50% cleavage, the inactive fraction rose rapidly. This resistance to inactivation at early reaction times indicated in Figure 4 does support the thesis that 5-Ag particles have a multiplicity of reactive sites. The experiments seem valid and fairly consistent among themselves. At extremes of no reaction and nearly complete reaction, the data indicate satisfactorily the expected responses; *i.e.*, virtually none in the inactive fraction and almost entirely all inactive.

Because reaction sites in the colnmn are extremely diluted, the fractionation should place material with binding energies lower than about 8 or 9 kcal in the inactive fraction. The concentration of cells in the column, approximately 100 times standard concentration, corresponds to an absorption limit of about 10^{-6} M absorption sites for 5-Ag. For an association constant of 10^6 M^{-1}, which corresponds to $-\Delta F_p^\circ$ of 8.3 kcal/mole, 50% or less of the component would be absorbed. This is an artificial cutoff point which is the result of the low concentration of absorption sites on the cells. Then component A with $-\Delta F_p^\circ$ less than the cutoff value will be inactive by this test. Relation to the model curves is discussed below.

[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.

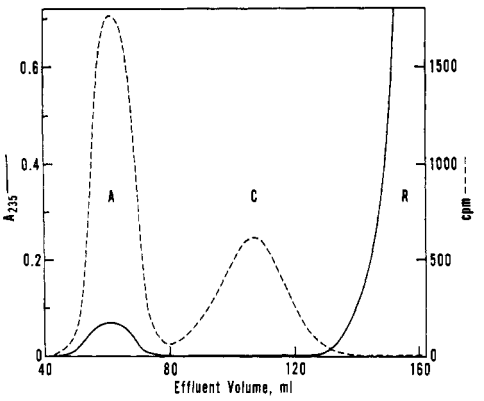

FIGURE 1: Resolution of the major component, A, and the 1.7S reduction fragment, C, of 5-Ag on Sephadex G-100. The data are from expt J (see text). Thirty-eight per cent of the recovered ^{35}S label appeared in component C. The reagent's peak, R, appears last and contains no ^{35}S label.

One experiment given in Figure 4 was performed using a larger 5-Ag preparation than the others (expt J, see Methods), to obtain greater precision and to lessen the possibility of losses during fractionation. The position of the data point in Figure 4, in agreement with the others, indicates that the experiments are valid and not the result of too much handling of a small amount of 5-Ag.

Absorption Isotherms of Component A and the Active Fractions. After the active fractions of component A were recovered by fractionation on type 21 cells, their absorption isotherms were determined. The values of $-\Delta F_p^\circ$ determined from these isotherms are given in Figure 5, including that for expt J. Evidently $-\Delta F_p^\circ$ values of the active fractions are much less than for the original 5-Ag, even though these fractions were selected by their specific binding activity. This observation establishes that the free energy of active 5-Ag can vary over a range of at least −11 to −14 kcal, and so is important in interpreting the behavior of 5-Ag.

Data corresponding to the association constants of unfractionated component A (Figure 2) are also given in Figure 5. After the experiments described in Figure 2 were performed, it was noted that some nonspecific absorption of the partially reacted component A occurred, which could be prevented by addition of phosphomannan to the medium. In all subsequent experiments, the phosphomannan was added. Since without phosphomannan, the binding energies appeared to be about 0.7 kcal higher, the experimental data on samples of component A given in Figure 5 are reduced by that amount. This correction is rather uncertain but unimportant because data from the active fractions are the more meaningful.

The curves describe the experimental data. The difference between them shows the contribution of inactive material in component A. The solid curve shows the reduction in $-\Delta F_p^\circ$ of active component A.

The absorption isotherm of the active fraction from expt J is shown in Figure 6. It is nearly linear, but as discussed

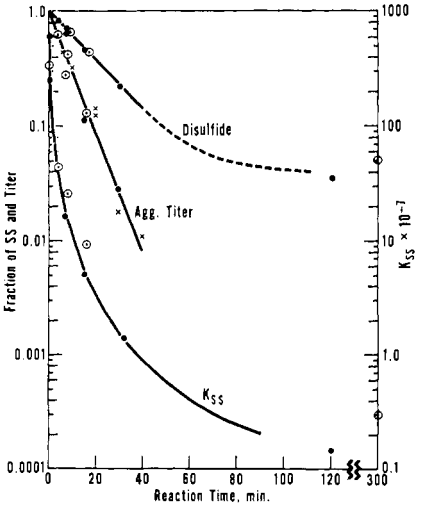

FIGURE 2: Variation of experimental parameters of 5-Ag during disulfide reaction at pH 6.7. The fraction of intact disulfide bonds, the fraction of agglutinative titer remaining, and the apparent association constants of component A, per mole of disulfide bonds in the original 5-Ag, are indicated. The different symbols refer to separate lots of 5-Ag.

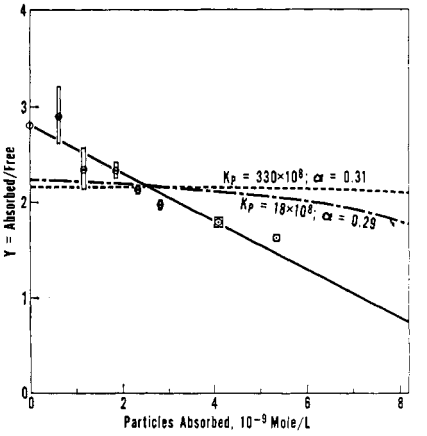

FIGURE 3: Absorption isotherm of component A from a preparation of 5-Ag reacted 15 min with mercaptoethanol. The solid line is the linear regression through the cell absorption limit (11.2×10^{-9} mole of particles/l.). The dashed curves are regressions on the data for theoretical behavior of 5-Ag of two components—one with an association constant, K_p, per mole of 5-Ag particle and the other, inactive (see text). Error boxes around the data points indicate the 34% probability range.

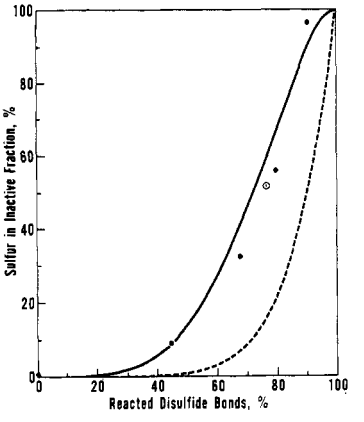

FIGURE 4: Variation of the percentage of sulfur in the inactive fractions of component A during the cleavage reaction of disulfide bonds. (●) Small-scale experiments; (⊙) expt J, a large-scale experiment. The solid curve shows the behavior of the theoretical model, described in the text, when single-site particles are in the inactive fraction; the dashed curve is for the same model when single-site particles are in the active fraction.

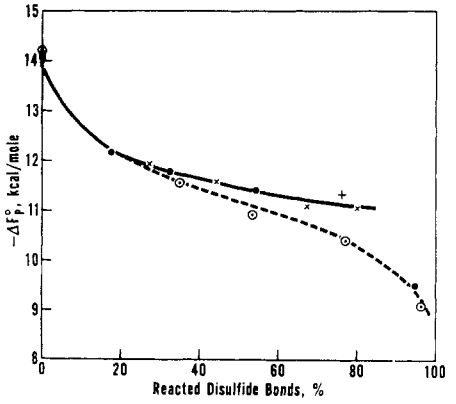

FIGURE 5: Variation of $-\Delta F_p^\circ$ for component A, and the active fractions of component A, with disulfide reaction. (●, ○) Component A, (×) active fractions, and (+) active fraction of expt J.

below this behavior does not preclude heterogeneity in the active fraction.

Absorption of the 1.7S Component by Type 21 Cells. The 1.7S fragments cleaved from the major component and separated on G-100 Sephadex were tested for absorption by complementary type 21 cells. This material is known to be only very weakly absorbed if at all (Taylor and Orton, 1968). In the first three lines in Table I specificity is examined. At rather high cell concentrations, a small but specific absorption of the 1.7S fragment occurs only on cells of the complementary mating type 21. Data are given as percentage absorbed, relative to radiolabel count in an equivalent suspension with cells of type 5. The two mating types of another sexually agglutinative yeast, *Saccharomyces kluyveri*, are not absorptive. Neither of these mating types absorbs 5-Ag either, so these cells can be considered as additional inactive controls.

The last two lines of Table I show absorption results for two special preparations of the 1.7S fragment in which precautions were taken to eliminate any contamination by active 5-Ag. The original 5-Ag was nearly completely inactivated and the C component fractionated on Sephadex

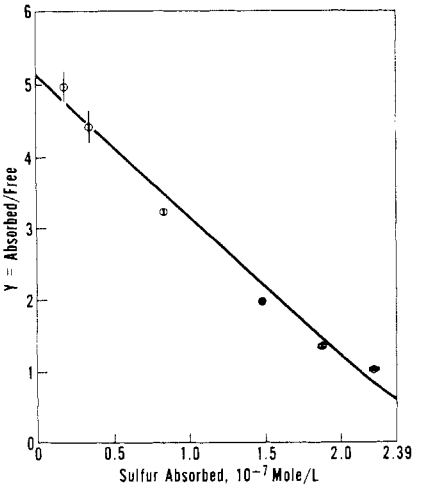

FIGURE 6: Absorption isotherm of the active fraction of component A from expt J. The curve is the theoretical isotherm for the system of components corresponding to the theoretical model described below when, as in expt J, 24% of the disulfide bonds are intact. The value of $-E$ was adjusted slightly to 5.54 kcal/mole to fit the data of this experiment.

TABLE I: Absorption of 1.7S Fragments.

Test Cells	Rel Cell Concn	Av % Absorbed
Specificity Test		
Type 21	25	5
Saccharomyces kluyveri type 3	25	1
S. kluyveri type 26	25	0
Contamination Test (Twice Purified 1.7 S)		
Type 21	120	10
Type 21	170	20

G-100. Then the column was washed thoroughly with solvent. After the C component was reduced in volume by ultrafiltration, it was repurified in the column to remove any traces of active 5-Ag. A significant fraction of these purified preparations of 1.7S fragment was still absorbed, which result demonstrates that the 1.7S fragment is indeed active for specific absorption, though absorption is very weak.

The low levels of absorption prevented our finding the absorption limits or the shape of the isotherms. Assuming the same limit per disulfide bond as for 5-Ag, values were calculated of approximately -7 to -8 kcal per mole for an estimate of the free energy of association of the 1.7S fragment. Since the absorption limit for 1.7S component would not likely be less than for the much larger and multivalent 5-Ag particles, this value represents a probable upper limit estimate of the magnitude.

Theoretical Model. A simple theoretical model can be constructed which describes the behavior of 5-Ag during reduction. Only a few reasonable *a priori* assumptions are necessary, and the model behavior can be tested in several different ways to decide whether it correctly describes 5-Ag.

The average 5-Ag particle is a large, major component containing a number of 1.7S fragments joined to it by disulfide bonds (Figure 7A). If the 1.7S fragments are distributed over the surface of the particle, then perhaps either one, two, or three neighboring active sites; *i.e.*, the 1.7S fragments, can combine with the complementary cell wall sites. We assume the wall does have enough active sites for this action. Since 5-Ag is agglutinative, not all the 5-Ag sites combine with one cell wall; presumably only about half or approximately three of them do so.

This formulation differs from the three-point binding enzyme complexes posed by Friess *et al.* (1962) in that the

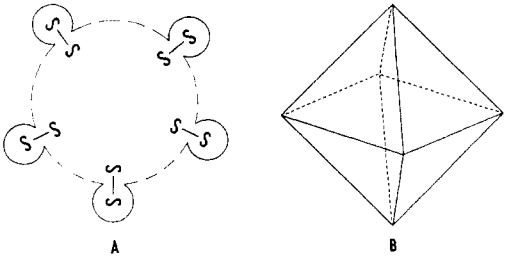

FIGURE 7: Models of 5-Ag. (A) Diagram of 1.7S fragments linked to the major component by disulfide bonds. (B) A regular octahedron; the suggested structure has an active site at each apex.

three or so complete, specific active sites are presumed to act independently but more or less additively.

The topological equivalent of a particle with six sites in which any two or three adjacent sites can be bound is represented by Figure 7B. Each of the active sites is represented by an apex; binding by two sites is along any edge; and binding by three sites is on any face. Even if the sites are distributed randomly on the surface, Figure 7B may reasonably represent the behavior.

Inspection of Figure 7B indicates that there are six ways of binding on one site, twelve on two sites, and eight on three sites. The partition function for these various combinations of binding energy is

$$K(6) = (6e^{-E/RT} + 12e^{-2T/RT} + 8e^{-3E/RT})\cdot Q \qquad (1)$$

where $K(6)$ is the apparent association constant of the particle with six intact sites, E is the unitary free energy of binding per mole of site, the term in parentheses is the derived partition function for binding energy, and Q is the remainder of the partition function.

By this formulation we wish to resolve the free energy of association into two components: (1) Q, corresponding to the free energy required to transfer the 5-Ag particles to the cell surface, which would include the cratic entropy term (Gurney, 1953) as well as any other nonspecific interaction energy between the particles and the cell wall; and (2) E, the unitary free energy of association for the individual sites. We assume E and Q are constant during cleavage of the disulfide bonds and temporarily we assume $Q = 1$ for a first approximation.

When the first disulfide bond is cleaved on such a 5-Ag particle, the contribution of that site as well as the four common edges (two-site groups) and the four common faces (three-site groups) are removed from the partition coefficient, leaving

$$K(5) = (5e^{-E/RT} + 8e^{-2E/RT} + 4e^{-3E/RT})\cdot Q \qquad (2)$$

In similar fachion, the partition coefficients for all other numbers of active sites, N, can be calculated, with proper allowance for the variety of combinations on particles with multiple inactive sites. This formula is admittedly somewhat over detailed. The important feature of any $K(N)$ is whether three-site interaction is allowed at all, etc. Otherwise the integer multipliers are of minor significance.

If all active sites are equivalent, the fraction of particles with N active sites, $P(N)$, can be calculated for any extent of disulfide reaction, from the binomial distribution for six events

$$P(N) = \frac{6!}{N!\cdot(6-N)!}\cdot(f)^N\cdot(1-f)^{6-N} \qquad (3)$$

where f is the fraction of disulfides remaining intact.

With the values of $K(N)$ and $P(N)$, an apparent average association constant, K_p, can be determined from

$$K_p = \frac{1}{\sum_N\left(\frac{P(N)}{K(N)+\frac{1}{L}}\right)} - \frac{1}{L} \qquad (4)$$

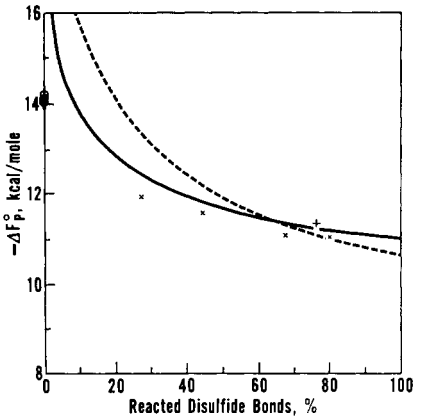

FIGURE 8: Variation $-\Delta F_p^\circ$ of the active fraction with disulfide reaction for the theoretical model. (——) Active fraction excludes single-site particles, $E = -5.5$ kcal/mole; (----) active fraction includes single-site particles, $E = -10.6$ kcal/mole. Data for experimental active fractions are repeated from Figure 5 for comparison. (○) 5-Ag, (×) active fractions of component A, and (+) expt J.

where L is the absorption limit of the cells (in the range 1–10 $\times 10^{-8}$ mole of particles/l. in our experiments). The constant is computed on the assumption that the absorption isotherm is linear up to the absorption limit, which gives K_p correct within about 20% for the model behavior discussed below, of the active fractions of component A. (Each $P(N)$ may contain several parts, each with a separate $K(N)$, owing to the variety of combinations of multiple inactive sites.) Then the theoretical average standard free energy of association

$$\Delta F_p^\circ = -RT\ln K_p \qquad (5)$$

can be computed.

Model Compared to Experiment. When none of the active sites remain on a 5-Ag particle, it is completely inactive. The fraction of such particles, $P(0)$, is given by eq 3. A small correction gives the fraction of sulfur in inactive particles, which corresponds to the dashed curve in Figure 4.

Experimentally, any 5-Ag particle with $-\Delta F_p^\circ < 9$ kcal/mole is placed in the inactive fraction. If $-E < 9$ kcal/mole, then 5-Ag particles which can only be bound at single sites are in the inactive fraction. This inactive fraction is given by $P(0) + P(1) + 0.2P(2)$. Corrected to sulfur basis, it is shown as the continuous curve in Figure 4. It is a better fit to the data than the former and indicates better agreement with weak, single-site interaction. No arbitrarily chosen parameters are required, aside from the initial assumptions in the model and the deduction from experiment of the 9-kcal cutoff.

In Figure 8 is shown the theoretical relationship between $-\Delta F_p^\circ$ of the active fraction and extent of disulfide bond reaction. Experimental data of the active fractions and of 5-Ag are repeated from Figure 5. The curves are computed to fit the data visually by finding an optimum value of E. For weak, single-site binding (solid curve), $E = -5.5$ kcal/mole. For strong, single-site binding (dashed curve), $E = -10.6$ kcal/mole.

A somewhat better fit to the data is found for the weak, single-site binding. The models also predict the sigmoid-type curve observed experimentally (Figure 5) for free energy of the total component A (theoretical curve not given).

The absorption isotherm derived from the weak, single-site model and corresponding to experiment J is shown in Figure 6. It is calculated from

$$Y = \frac{1}{\sum_N \left(\frac{P(N)}{K(N)\cdot(L - X) + 1} \right)} \quad (6)$$

where Y is the absorption ratio, L is the absorption limit, and X is the molar concentration of particles absorbed.

For computation purposes, the values X_s and Y_s based on molar sulfur concentrations were found from the equations

$$Y_s = \frac{1}{\sum_N \left(\frac{P(N)\cdot S(N)}{K(N)\cdot(L - X) + 1} \right)} - 1 \quad (7)$$

and

$$X_s = X \cdot \frac{\sum_N (P(N)\cdot S(N)) - \sum_N \left(\frac{P(N)\cdot S(N)}{K(N)\cdot(L - X) + 1} \right)}{1 - \sum_N \left(\frac{P(N)}{K(N)\cdot(L - X) + 1} \right)} \quad (8)$$

where $S(N)$ is the number of sulfur atoms in particles with N intact bonds. The plot of Y_s *vs.* X_s is shown in Figure 6, calculated for an intact disulfide-bond fraction, 0.238, observed in expt J.

With only a slight adjustment of E, to -5.55 kcal/mole, the theoretical isotherm fits the data quite adequately. The strong, single-site model also gives an acceptable fit with E adjusted to -10.7 kcal/mole, so no choice between the alternatives is available for this test, but consistency with the overall model is shown.

The slight concavity upward shown by the line and the data (other experiments confirm it) is the result of the diminished sulfur content of the more weakly bound particles. This special complication has produced the opposite of the downward concavity anticipated for heterogeneous absorbates.

The cell absorption limit for 5-Ag in expt J was 3.38×10^{-7} mole of sulfur per l. Correction for the decreased average sulfur content of the active fraction of component A gives a limit of 2.39×10^{-7} mole/l. The data appear to extrapolate fairly closely to this limit for practical purposes. The approximate calculations of ΔF_p° (Figure 8) which are based on this limit are then quite satisfactory.

Relation to Agglutination. Agglutinative titer falls rapidly during cleavage of the disulfide bonds in 5-Ag. Comparison to the model (not given) indicates titer to be proportional to the number of five- or six-site 5-Ag particles remaining, such as would be expected if three sites are required for strong binding. This comparison can hardly be definitive, since agglutinative titer decline occurs early in disulfide cleavage, where only the average disulfide count per particle is known. It does indicate, though, that the model is adequate in indicating this fast decline in titer.

Value of Q. The possible range of values for Q in eq 1 can be considered. The most reasonable assumption is the usual one for associative reactions—that the free energy should be corrected for an entropy of dilution applied once for each particle. The unitary free energy (Karush, 1962; Kauzmann, 1959; Gurney, 1953) is the standard free energy of association after correction for this entropy term, which is $T\cdot\Delta S = -2.4$ kcal/mole at room temperature. The binding energy, E, in eq 1 should then be the unitary free energy per single binding site. The entropy correction is reasonable, but its magnitude is somewhat uncertain; also, other factors such as nonspecific attractive and repulsive forces may contribute to Q. Such forces would be expected to be only moderate to weak though. When all these contributions are considered, a range of values from 0 to -6 kcal per mole should cover the reasonably possible range of $RT \ln Q$.

Over the range of $RT \ln Q$ as given, the behavior of the model is similar to that described in Figure 8 for $Q = 1$, *i.e.*, $RT \ln Q = 0$. As $RT \ln Q$ becomes more negative, the $-\Delta F_p^\circ$ curve becomes somewhat higher at low disulfide reaction but otherwise is about the same as the curve shown. The value of $-E$, which was chosen to be 5.5 kcal/mole for $RT \ln Q = 0$, becomes 6.5 kcal/mole for $RT \ln Q = -2.4$ kcal/mole, and becomes 8.3 kcal/mole for RT in $Q = -6$ kcal/mole. Thus, we can conclude that for any reasonable value of Q, the model can agree with experiment by suitable choice of one arbitrary parameter. As a corollary, we also conclude that because this term is uncertain, the value of $-E$ is also rather uncertain, but likely it lies in the range 5–9 kcal/mole for the weak, single-site model.

Discussion

Validity of the Model. A model for the behavior of 5-Ag during progressive removal of the active sites has been formulated. If we assume that each 1.7S fragment has an active site, and that $-E$ is less than about 9 kcal/mole, then this model agrees with experimental data in six ways. (1) The average $-\Delta F_p^\circ$ of component A decreases in a sigmoid curve with increasing disulfide-bond cleavage. (2) The average $-\Delta F_p^\circ$ of the active fraction of component A decreases sharply with low levels of disulfide cleavage. (3) The inactive fraction in component A remains negligible until about 30% disulfide cleavage, and then rises sharply. (4) The absorption isotherms of active fraction of component A show no indication of heterogeneity. (5) The 1.7S fragments are weakly but specifically bound to cells of complementary mating type. (6) Rapid decline in agglutinative titer is predicted.

Of these six coincidences, points 1, 4, and 6 are merely checks for consistency. The remaining three coincidences, while not individually proving the model, together strongly suggest that 5-Ag is structured similar to the model. The model's better description of the fraction of inactive sulfur (Figure 4), and the somewhat better description of $-\Delta F_p^\circ$ (Figure 8) favor the weak, single-site alternative.

The experimental estimate of $-\Delta F^\circ$ for the 1.7S fragment, 7 kcal/mole or less, lies in the same range as our estimates of the negative of unitary free energy per site on 5-Ag, 5.5 kcal/mole for the weak-site model. Experimental difficulties and the uncertain contribution of Q prevents a closer comparison, but these results also favor the weak, single-site alternative.

Structure of 5-Agglutinin. We now believe that the individual active sites of 5-Ag are weak. Our previous conclusion that the 1.7S fragments were not the active sites was based on the very low absorptive activity of this component. When Brock (1959) first examined the chemistry of the sexual agglutination reaction in yeast, he suggested that the sites on mating type 21 might be protein and those on type 5 might be carbohydrate. Since then, though we have tried to determine the nature of the active site and have suggested possible structure components (Brock, 1965; Crandall and Brock, 1968; Taylor and

Orton, 1968), it is evident that the sensitivity of the disulfide bonds joining the active sites to the 5-Ag particle and the weakness of the individual sites have obscured the true nature of the sites themselves and continue to do so. We still have almost no information on their active structures.

We do know that protein is an important part of the structural matrix which holds the active agglutination sites on cells of mating type 5. Proteases can liberate 5-Ag from the cells, and can also reduce the size of the large ($M = 10^8$) agglutinative particles obtained by treating cells with snail enzyme, without a large drop in agglutinative activity. The presence of disulfide bonds on 5-Ag also suggests a protein structure, and the correspondence of 1 bond per 1.7S fragment suggests that this protein may be part of the unique structure at the active site, though not necessarily the active site itself.

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Inhibition of Mitochondrial Energy-Linked Functions by Arsenate. Evidence for a Nonhydrolytic Mode of Inhibitor Action*

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ABSTRACT: Intact rat liver mitochondria and sonicated particles from beef heart mitochondria catalyze a prominent arsenate $\rightleftharpoons$ water exchange in the absence of an energy source. Unlike the phosphate $\rightleftharpoons$ water exchange, the arsenate $\rightleftharpoons$ water exchange reaction does not require ADP. These observations are in line with previous findings on the insensitivity of arsenate $\rightleftharpoons$ water exchange to dinitrophenol and oligomycin and provide no evidence to support the idea that the exchange is a partial reaction of oxidative phosphorylation indicative of arsenate activation to form, for example, a covalent high-energy arsenyl derivative with enzyme or with ADP. Two distinct modes for arsenate inhibition of energy-linked functions were noted. Arsenate inhibition of phosphate-requiring reactions (oxidative phosphorylation and phosphate $\rightleftharpoons$ ATP exchange) was competitive with phosphate as expected.

However arsenate inhibition of energy-linked reduction of NAD^+ by succinate (driven by ATP) was not relieved by phosphate. Phosphate and arsenate exerted identical inhibitory effects on energy-linked reduction and both compounds required the presence of ADP for their inhibitory effects to be displayed. The first mode of inhibition (of phosphate-requiring reactions) does not require the postulation of an arsenolytic reaction and the second mode of inhibition (of energy-linked reduction) is at variance with this type of arsenate induced hydrolytic action.

Arsenate (As_i)[1] has been shown to substitute for phosphate in the stimulation of glycolysis by soluble yeast extracts (Harden and Young, 1906). Arsenate, unlike phosphate, was not incorporated into organic form. It was later suggested that the effect of arsenate on glycolysis was associated with an uncoupling of an oxidative reaction from ATP synthesis at the triose phosphate level (Needham and Pillai, 1937).

This proposal was substantiated by the demonstration that arsenate stimulated the oxidation of glyceraldehyde 3-phosphate in the absence of phosphate (Warburg and Christiansen, 1939). These findings led to the concept of "arsenolysis" to describe the hydrolytic breakdown of an unstable arsenyl analog of a naturally occurring phosphoryl compound (Doudoroff *et al.*, 1947). Early studies of the effect of arsenate on mitochondrial systems showed that relatively high concentrations of arsenate stimulated respiration and decreased phosphorylation (Crane and Lipmann, 1953). A stimulation by arsenate of mitochondrial ATPase was investigated using arsenate labeled with ^{18}O (Itada and Cohn, 1963). As a control,

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: As_i, inorganic arsenate; DNP, 2,4-dinitrophenol.