Molecular Sieve Studies of Interacting Protein Systems. V. Association of Subunits of D-Amino Acid Oxidase Apoenzyme*

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ABSTRACT: The association of subunits of hog kidney D-amino acid oxidase apoenzyme was studied by analytical molecular sieve chromatography using the method of integral boundary experiments. The degree of subunit association was determined as a function of protein concentration over the range 3–900 μg/ml at eight temperatures between 4 and 20°. Over this range of conditions the apparent molecular weight as determined from column calibration increased with concentration and assumed values intermediate between monomer and dimer. The stoichiometry of association which provided a best fit to the experimental parameters was that of a dimerization reaction. The apparent dimerization constant was calculated at each temperature from the experimentally determined weight-average partition coefficients measured at a series of protein concentrations. Investigation of the temperature dependence of subunit dimerization revealed a marked transition in the value of the association constant in the region of 12–14°, with the dimerization proceeding more favorably at temperatures above 14° and less favorably at temperatures below 12°. The molar enthalpy change for the association on either side of this transition was found to be approximately zero, but a value of 72 kcal/mole was found for ΔH over the transition range (12–14°).

The pronounced variation of ΔH with temperature indicated a reversible change in heat capacity for the protein and strongly suggested that the protein might be undergoing isomerization reactions between different conformational states in addition to subunit association. Analysis of expected behavior for a system undergoing simultaneous dimerization and isomerization of subunits leads to the prediction that any measurement (*e.g.*, optical spectra) capable of reflecting differences between different conformational isomers should exhibit concentration dependence at constant temperature. Evidence for the existence of such conformation isomers was found in the concentration dependence of the ultraviolet absorption, and optical rotatory dispersion, spectra of the protein.

Since subunit association reactions are often closely related to the functional state of a protein complex and to the binding of regulatory molecules, it is important to know the conditions under which a given level of association might be expected. The enzyme D-amino acid oxidase (D-amino acid: oxygen oxidoreductase (deaminating), EC 1.4.3.3) has been shown to be such an associating system in which the physical properties of the protein are affected by coenzyme and substrate (Charlwood *et al.*, 1961; Antonini *et al.*, 1966; Henn and Ackers, 1969). Recent studies have revealed a sharp transition in catalytic activity and in optical properties of the holoenzyme in the region 12–14° which were attributed to a temperature-dependent structural transition in the protein (Massey *et al.*, 1966). This apparent conformational change is also reflected in the subunit association equilibria. At constant protein concentration the molecular weight of holoenzyme has been found to increase with temperature over the transition region, as indicated by light scattering (Antonini *et al.*, 1966). However, this temperature dependence was not observed in the light scattering of apoprotein, prepared by removal of coenzyme (FAD) from holoenzyme. Therefore, it was not clear to what extent the temperature-dependent transition in physical properties observed for the holoenzyme might be the result of coenzyme binding as opposed to protein conformational change.

One possibility is that the results obtained with holoenzyme are partially or entirely the result of temperature dependence in FAD binding. However, as pointed out by Massey *et al.* (1966) the high molar enthalpy change which accompanies the transition (78 kcal/mole) would appear indicative of some protein conformational change, rather than coenzyme binding alone. A second possibility, is that the binding of FAD in some way "sensitizes" the protein to undergo the temperature-dependent structural transition (for example, by a conformation change associated with binding). A third possibility is that the temperature-dependent transition for the (relatively unstable) apoenzyme was masked in the light-scattering measurements by the presence of large amounts of denatured material and that FAD binding stabilizes the protein against irreversible denaturation.

In the present study we have investigated the temperature dependence of apoenzyme association in the concentration range 3–900 μg/ml, using analytical molecular sieve chromatography. Results of this study indicate that substantial amounts of denatured protein are formed slowly and irreversibly under the solvent conditions employed in previous studies of this enzyme. The denatured component is easily detected chromatographically and can be corrected for in the elution patterns. It is then found that the remaining apoprotein undergoes association equilibria which exhibit a sharp transition in the region 12–14° as found previously for the holoenzyme and benzoate complex. Thermodynamic characterization of the subunit association equilibria strongly suggests the existence of simultaneous equilibria between different conformational states of the apoprotein. Optical measurements have

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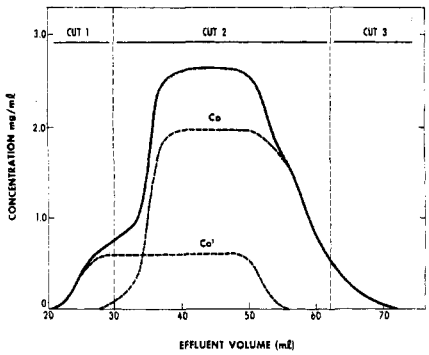

FIGURE 1: Elution profile of D-amino acid oxidase apoprotein chromatographed on Sephadex G-100. A 30-ml sample at a concentration of 2.64 mg/ml was applied to the column (1.2 × 60 cm) jacketed at 20°. The noninteracting component is seen as a shoulder on the measured elution profile (solid line). The elution diagram was resolved by a procedure of boundary analysis (see text) into contributions of the noninteracting component (of plateau concentration C_0') and associating components (plateau concentration C_0) shown by the dashed lines.

been carried out which provide evidence for these isomerization equilibria.

Experimental Procedures

Materials. The following proteins were used for column calibration: bovine γ-globulin (Cohn, Fxn II, lot 15B-2920) from Sigma; crystalline bovine serum albumin (lot 186 from Poviet Producten, Amsterdam); ovalbumin (five-times recrystallized, 6443) from Nutritional Biochemical Corp. and from Sigma (17B-8691); crystalline sperm whale myoglobin (J1381); bovine pancreas trypsin (twice recrystallized); horse heart cytochrome *c* (R3282) and hog stomach pepsin (three-times recrystallized) from Mann Research Laboratories. Blue Dextran (Pharmacia, TO-4474) and the Sephadex gels G-75 (lot 7118) and G-100 (lot 8080) were obtained from Pharmacia Fine Chemicals. All other reagents used were analytical grade. The buffer used in all studies was 0.1 M sodium pyrophosphate-HCl (pH 8.5).

Preparation of D-*Amino Acid Oxidase Apoenzyme.* D-Amino acid oxidase was purified from hog kidney as described previously by using the procedure of Kubo *et al.* (1958) as modified by Massey *et al.* (1961), and by removing the high molecular weight impurities by column chromatography on Sephadex G-75 (Henn and Ackers, 1969). All experiments were carried out with these highly purified preparations from which the nonenzyme protein contaminants had been removed (see Henn and Ackers, 1969, for details).

Analytical Chromatography. Columns (1.2 × 60 cm) were constructed as described previously for analytical determinations (Ackers and Thompson, 1965). The bottom of each column was connected to flexible polyethylene tubing leading to a microflow cuvet (0.2-ml volume, 1-cm path length with quartz windows). The effluent was monitored at 220 or 280 mμ, depending upon the protein concentration, by means of a Gilford 2000 multiple-sample absorbance recorder. Effluent volumes were measured using a 50-ml buret into which outflow from the flow cell was led. Volume readings could thus be made to within ±0.05 ml. Reproducibility of elution volumes, V_e, determined from peak position measurements of calibrating proteins was ±0.15 ml. Columns were washed with buffer for 24 hr prior to use. Temperature control of the jacketed columns was maintained with a Tamson water bath (Neslab Instruments).

Partition coefficients, σ, of the calibrating standards were determined from the measured elution volumes, V_e, according to the equation

$$\sigma = \frac{V_e - V_0}{V_i} \tag{1}$$

The void volume, V_0, was determined as the elution volume of Blue Dextran ($\sigma = 0$) and V_i, the internal volume, was similarly determined using potassium chromate ($\sigma = 1$). Columns were calibrated according to methods previously described for molecular radius (Ackers, 1967b) and molecular weight (Andrews, 1964). In the microgram per milliliter concentration range the apoenzyme has been shown to dissociate to a subunit with molecular radius of 25 Å and molecular weight of 35,000–40,000 (Henn and Ackers, 1969). In order to characterize quantitatively this association the method of integral boundary measurements was used (Ackers and Thompson, 1965; Ackers, 1967a). A sufficiently large sample of protein solution is applied to the column to establish a plateau in the elution profile of concentration *vs.* volume. Then a centroid elution volume

$$\bar{V} = \frac{\int_0^{C_0} V dC}{C_0} \tag{2}$$

is determined by planimetry across the leading boundary. In eq 2, C is protein concentration within the boundary, C_0 the plateau concentration, and V is volume. The centroid volume, $\bar{V}$, provides a measure of the weight-average partition coefficient

$$\bar{\sigma}_w = \frac{\Sigma \sigma_j C_j}{\Sigma C_j} \tag{3}$$

Individual partition coefficients σ_j pertain to various aggregates (j-mers) and the C_j terms represent their respective concentrations in the plateau region of total concentration, C_0. The weight-average partition coefficient, $\bar{\sigma}_w$, is calculated for each run from the measured centroid elution volume, $\bar{V}$, the void volume, V_0, and internal volume, V_i, by the relationship

$$\bar{\sigma}_w = \frac{\bar{V} - V_0}{V_i} \tag{4}$$

As the total concentration (ΣC_j) is increased, the various equilibria are shifted to favor the more highly aggregated species and this change is reflected in $\bar{\sigma}_w$ through the relationship given in eq 3. The experimental determination of $\bar{\sigma}_w$ as a function of plateau concentration C_0 ($= \Sigma C_j$) therefore provides an association curve which reflects the changes in level of subunit association with concentration.

Optical Studies. Concentration difference spectra were obtained at 20° according to the method of Fisher and Cross (1965) using a Cary 14 spectrophotometer equipped with a

0.0-0.1-optical density slide-wire. Quartz cylindrical cells of 1-, 2-, 5-, and 10-cm path lengths were used with the longer path-length cell in any particular experiment in the reference compartment.

Optical rotatory dispersion was measured at 27° with a Cary Model 60 spectropolarimeter.

Results

Elution Profiles. The elution profiles obtained in the integral boundary experiments exhibited the type of concentration-dependent transport behavior expected for an associating system which contains an additional noninteracting component (Timasheff and Townend, 1960). An example of an elution profile which illustrates the presence of this component is shown in Figure 1. The additional component produces a shoulder on the leading edge at the void volume as shown in Figure 1. This shoulder is followed by a rise to the plateau. Following the plateau, the trailing edge shows a corresponding (although less pronounced) drop equal to the apparent height of the shoulder on the leading edge. This drop is followed by the broad trailing edge characteristic of an associating system (Ackers, 1967a). When the plateau concentration was varied, the position of this shoulder was found to remain constant, whereas the rest of the zone was found to exhibit concentration-dependent behavior expected for an associating system, *i.e.*, decreasing the plateau concentration resulted in a shift of the zone toward larger elution volumes.

It was initially thought that the leading shoulder might be the result of a relatively slow reversible interaction between molecular species such that the rate of transport through the column is rapid in comparison with the rate of the reaction. However, the presence and configuration of the shoulder profile was found not to depend upon flow rate of the column. Furthermore, it was found that the shoulder material, when separated off, diluted, and rerun, always appeared at the void volume and could not be converted into the material of larger elution volume, even after prolonged periods of time. These characteristic features are illustrated by the solute zone shown in Figure 1 from which three successive cuts were made. Cut 1 was the shoulder part of the leading edge, as indicated. Cut 2 was the rest of the leading edge and extended halfway down the trailing edge. Cut 3 was the tail end of the trailing edge. Cut 1 was diluted to a concentration of 28 μg/ml and allowed to sit in the cold room for 84 hr. It was then rerun under identical conditions and was found to elute at the same position as the more concentrated sample, *i.e.*, at the void volume. Since dilution did not result in dissociation even after prolonged periods of time, it was concluded that the shoulder material represented a truly nondissociating component. It was noted that formation of this component was favored by high temperatures or pressures, high protein concentrations, and storage over prolonged periods of time. It thus appeared to be an irreversible aggregate of D-amino acid oxidase apoenzyme. Furthermore, enzyme activity assayed at four points across the zone of the shoulder material when rerun (Figure 1, cut 1) showed an average specific activity of only 1.3 μmoles of pyruvate oxidized per min per mg of protein as compared with a specific activity of 12 μmoles of pyruvate oxidized per min per mg of protein for the trailing edge (cut 3).

When cut 3 was rerun it gave the elution profile shown in Figure 2, in which no shoulder is observed. It was thus possible to remove the shoulder material completely by chromatography and to obtain elution diagrams of the associating components only. However, it was not possible to prevent re-formation of the shoulder material in reconcentrating the apoprotein (*e.g.*, by pressure dialysis) for further runs. An alternative procedure was therefore developed in which the contribution of the shoulder material to the elution profile could be estimated with reasonable accuracy (±5%) and then subtracted.

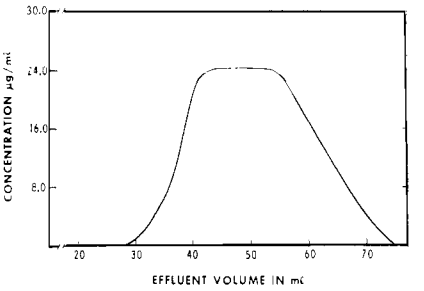

FIGURE 2: Elution pattern of cut 3 shown in Figure 1, diluted to 30 ml and applied to the same column at a concentration of 26 μg/ml. The high molecular weight noninteracting component has been completely removed.

Evaluation of Percentage Noninteracting Component. Calculations were performed to subtract the contribution of the noninteracting protein component to the elution profile and thus to resolve the measured curve into two profiles, pertaining, respectively, to the noninteracting component and to the associating species. The amount of noninteracting component to be substracted was evaluated by a procedure based on the expected shape of the leading boundary from chromatographic theory (Ackers, 1967a; Houghton, 1964). The shape for a single component or for a mixture of components all eluting at the void volume is a gaussian error function.[1] Thus on the leading edge of the shoulder where it can be assumed that only noninteracting component is present, the concentration–volume profile can be represented by the equation

$$C_T = C_0' \mathrm{erf}\left[\frac{V - V_0}{\delta}\right] \tag{5}$$

where C_T is the concentration pertaining to the volume V, V_0 is the void volume, and δ is a constant which defines the boundary spreading. C_0' is the plateau concentration of the noninteracting component. V is the volume coordinate of the elution diagram and erf is the error function. Rearranging this equation, we have

$$V = V_0 + \mathrm{erf}^{-1}\left[\frac{C_T}{C_0'}\right] \cdot \delta \tag{6}$$

This relationship provides a means of evaluating C_0' since a plot of volume *vs.* the error function inverse of C_T/C_0' is linear whenever a correct value of C_0' is chosen. Beginning with

[1] A gaussian form cannot be assumed for the leading edge of the interacting components, due to the boundary-sharpening effect (Ackers, 1967a). Equation 5, which neglects column "end effects," is a good approximation for the conditions of the present experiments.

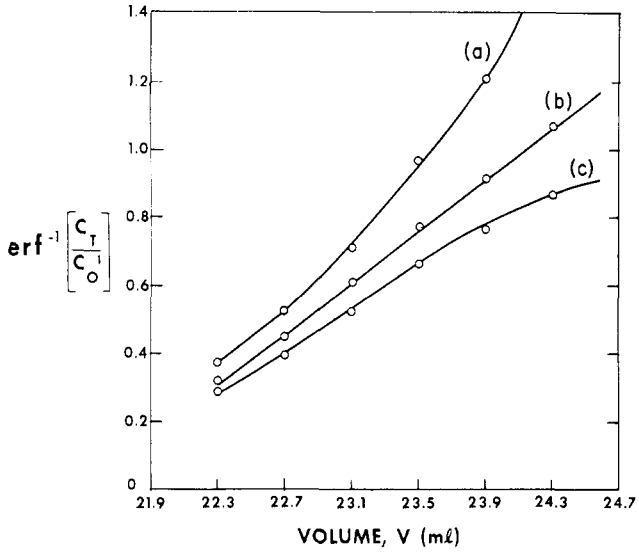

FIGURE 3: Evaluation of percentage noninteracting component according to eq 5 for the elution diagram shown in Figure 1. The iterative procedure consists of finding a value of C_0' which gives a linear plot. The curves shown here are for three values of C_0': (a) 0.561 mg/ml, (b) 0.636 mg/ml, and (c) 0.710 mg/ml. The value of 0.636 represents the best fit obtained for these data.

an initial choice of C_0' made "by eye" the correct value is determined by iteration as the value which provides the best linear plot of V against the inverse error function of (C_T/C_0'). The procedure is illustrated in Figure 3 for the elution profile shown in Figure 1 and the resolution of this elution diagram into two diagrams is shown by the dashed lines in Figure 1. The accuracy of this subtraction procedure was found to be $\pm 5\%$. After the correction has been made, the centroid volume, $\bar{V}$, of the leading boundary can be determined for the associating protein, and used to determine $\bar{\sigma}_w$, the weight-average

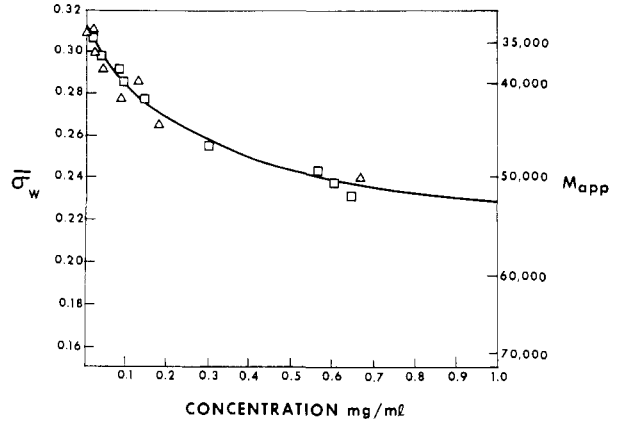

FIGURE 4: Association curve for D-amino acid oxidase apoprotein. The weight-average partition coefficients were determined as a function of concentration from the centroid elution volumes of the leading boundaries of the solute zones. The points represent experimental values and the curves are calculated for a dimerization reaction with equilibrium constant $K_D = 3.3$ ml/mg. (□) Data at 10° and (△) data at 4°. Corresponding values of the apparent molecular weight, M_{app}, were determined for each $\bar{\sigma}$ from the empirical calibration for molecular weight (eq 6).

TABLE I: Equilibrium Constants for Apoenzyme at 10°.[a]

C_0 (mg/ml)	V_e (ml)	$\bar{\sigma}_w$	α	K (ml/mg)
0.017	34.4	0.309	0.961	2.5
0.034	37.9	0.299	0.895	3.9
0.082	37.55	0.292	0.849	2.6
0.091	39.1	0.285	0.806	3.3
0.147	38.75	0.278	0.761	2.8
0.300	37.5	0.255	0.613	3.5
0.566	35.0	0.243	0.526	3.0
0.603	35.7	0.236	0.490	3.5
0.648	34.4	0.231	0.447	4.3

[a] Average $K = 3.3 \pm 0.6$ ml per mg.

partition coefficient of the associating species according to eq 3.

It was found that, for a given plateau concentration, C_0 (corrected for nondissociating component), the value of $\bar{\sigma}_w$ was independent of the amount of shoulder material that had been formed. This result indicates that the shoulder material is truly noninteracting. Not only is it nondissociable but it does not interact with the remaining components of the protein sample.

Stoichiometry of Association. At a given temperature the values of $\bar{\sigma}_w$ provide an association curve which reflects primarily the change in average molecular weight with C_0. A representative curve is shown in Figure 4 for data obtained at 10°. A typical set of results is shown in Table I for integral boundary experiments at 10° over a concentration range of 0.017–0.65 mg/ml. Series of integral boundary experiments were also done at 20°, over a 300-fold concentration range from 0.003 to 0.9 mg per ml, and at 4° over a 37-fold concentration range from 0.018 to 0.67 mg per ml. For the other temperatures studied a large sample of apoenzyme, free of nonenzyme protein contaminants, was prepared and experiments were done in duplicate with plateau concentrations ranging between 35 and 55 μg per ml. The values of $\bar{\sigma}_w$ were found to increase with concentration between the partition coefficient values for monomer and dimer. The partition coefficient of monomer was determined by extrapolation of $\bar{\sigma}_w$ to infinite dilution. From data obtained at these extremely low concentrations (down to 3 μg/ml) the extrapolation can be carried out with accuracy to within a few per cent in the estimation of σ_1. The partition coefficient of dimer was estimated by two different approaches. (a) *From the empirical molecular weight calibration* of the Sephadex G-100 gel. Based on the calibrating standards used (see Materials) the relationship between molecular weight, M, and partition coefficient, for the G-100 can be expressed as

$$\sigma = -A \log M + B \qquad (7)$$

Using this relationship values of the partition coefficient for dimer of $M = 70{,}000$ (Henn and Ackers, 1969) were calculated. For each experimental value of $\bar{\sigma}_w$ an apparent molecular weight, M_{app}, may also be calculated from eq 7, as illustrated in Figure 4. For low levels of association such as these, M_{app}

can be shown to approximate the weight-average molecular weight (G. K. Ackers, unpublished results). (b) Partition coefficients of dimers were also estimated *from the relationship between molecular Stokes radius, a, and partition coefficient* (Ackers, 1967b).

$$\sigma = \text{erfc}\left[\frac{a - a_0}{b_0}\right] \tag{8}$$

The calibration constants a_0 and b_0 were determined from the measured partition coefficients of the standard proteins as described previously (Henn and Ackers, 1969). This relationship can be used to estimate partition coefficients of *n*-mers taking into account the possible contributions of molecular shape. In the present study molecular radii, a_n of *n*-mers, were calculated for two extreme modes of aggregation: (1) *Compact aggregation* in which both monomer and *n*-mer are assumed to be spheres and the molecular asymmetry is minimum. For this limiting case a_n is related to a_1 by the relationship

$$a_n = a_1 n^{1/3} \tag{9}$$

For example, in the case of dimer ($n = 2$), a_2 is calculated from a_1 (determined by eq 8 from σ_1 and the calibration constants a_0 and b_0) as

$$a_2 = (24.9 \text{ Å})(2^{1/3}) = 31.4 \text{ Å}$$

(2) The second limiting case is that of *linear subunit aggregation* in which the asymmetry of *n*-mer is maximum. For this model a_2 is calculated as the Stokes radius for an ellipsoid having frictional ratio f/f_0 equal to that of a cylinder for which the length to diameter ratio is *n*. For such a model the axial ratio, ρ, is related to the ratio of length, L, to diameter, d, by the expression (Tanford, 1961)

$$\rho = \left(\frac{2}{3}\right)^{1/2} \frac{L}{d}$$

and the corresponding frictional ratio $(f/f_0)_n$ may be determined from ρ by the Perrin equation (tabulated values given in Schachman, 1959). Then for this linear aggregation model

$$a_n = a_1 n^{1/3} \left(\frac{f}{f_0}\right)_n \tag{10}$$

For dimer $\rho = 1.632$ and the Stokes radius of dimer becomes

$$a_2 = (24.9)(1.26)(1.028) = 32.2 \text{ Å}$$

The partition coefficient of dimer calculated fron a_2 by eq 8 for the two extreme limiting cases were found to differ by 8%. Thus it was determined that an 8% uncertainty exists in the determination of σ_2. A similar estimation of limits for σ_3 provided an uncertainty of 15%. Taking into account these limits of uncertainty it was possible to distinguish between monomer–dimer and monomer–trimer models on the basis of the experimental results in the following way. For a monomer *n*-mer reaction eq 3 can be expressed in the form (Ackers and Thompson, 1965).

$$\bar{\sigma}_w = \alpha\sigma_1 + (1 - \alpha)\sigma_n \tag{11}$$

where α is the weight fraction monomer. Then

$$\alpha = \frac{\bar{\sigma}_w - \sigma_n}{\sigma_1 - \sigma_n} \tag{12}$$

and the equilibrium constant, K, is given by

$$K = \frac{(1 - \alpha)}{\alpha^n C_0^{n-1}} \tag{13}$$

Values of α are calculated at each C_0 by eq 12 using the experimental values of $\bar{\sigma}_w$ and σ_1 and the estimated values of σ_n. The equilibrium constant may then be calculated by eq 13. The test for consistency of a given model with the data then lies in determining whether a systematic variation with concentration exists in the calculated values of K.

It was found that a dimerization model provided a good fit over the range of conditions employed in this study, but monomer–trimer and monomer–tetramer models did not. Some representative values are tabulated for the 10° data in Table I. These results indicate that the association can be characterized as a dimerization rather than a reaction of higher stoichiometry. They do not rule out completely the presence of other species. In fact, higher order reactions may occur at concentrations above those studied and are indicated by deviations from the monomer–dimer curve at a concentration of 0.9 mg/ml at 20°. The limitations of this type of analysis have been critically discussed (Ackers, 1968). Within these limitations the results obtained in the present study can be taken as a strong indication that the predominant species involved at concentrations below 0.7 mg/ml are monomer and dimer, and that the constant K calculated from the data by eq 13 closely approximates the dimerization constant for the system.

Temperature Dependence of Subunit Association. At a given concentration of protein, C_0, the level of subunit association was found to be markedly temperature dependent, as reflected in the experimentally determined values of $\bar{\sigma}_w$ and the calculated dimerization constants. The most extensive measurements were carried out at 20, 10, and 4°. The mean value for the dimerization constant calculated from 13 determinations at 20° was 8.8 ±3.9 ml per mg, whereas, the mean value of nine determinations at 10° was found to be 3.3 ± 0.6 ml per mg and that for eight runs at 4° was 3.2 ± 1.3 ml per g. Additional values of $\bar{\sigma}_w$ were determined in duplicate experiments at each of six temperatures between 8 and 20°. For these determinations a concentration range (35–55 μg/ml) was chosen in which a large difference in $\bar{\sigma}_w$ values had previously been found between the 10 and 20° data. The apparent dimerization constant, K_D, at each temperature was calculated by

$$K_D = \frac{(1 - \alpha)}{\alpha^2 C_0} \tag{14}$$

at each plateau concentration. The results are summarized in Table II. It was found that these equilibrium constants fell

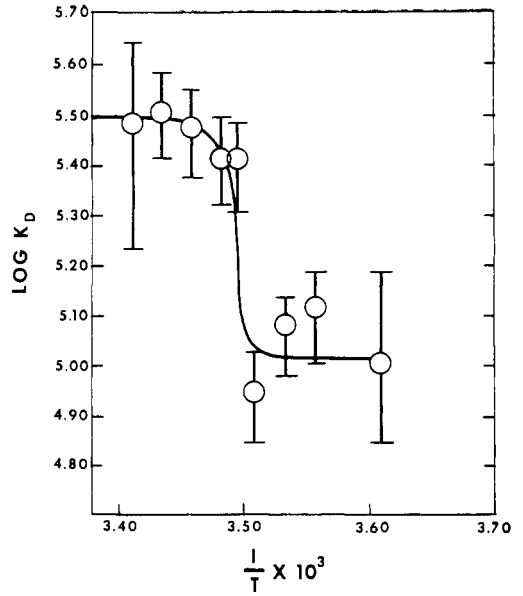

FIGURE 5: Van't Hoff plot for the dimerization of purified apoenzyme at temperature of 4–20°. It is seen that the protein undergoes a sharp thermal transition in the regions 12–14°. The molar enthalpy change, represented by the slope of this plot, was calculated over the transition range to be 72 kcal/mole. The levels of uncertainty indicated for values of log K_D represent the uncertainties in the estimation of partition coefficients for dimer as well as the inaccuracy in determination of $\bar{\sigma}$ values.

into two classes depending upon temperature. At temperatures of 14° and greater the protein dimerizes with an equilibrium constant of approximately 8 ml/mg, whereas, at temperatures of 12° and below K_D is less than half this value (*ca.* 3 ml/mg). The association reaction is, therefore, endothermic, proceeding more favorably at higher temperatures than at lower ones. The sharp transition in the affinity between subunits in the region of 12–14° is reflected in the values of K_D. It thus appears that at temperatures below this transition region one thermodynamic state of the protein is more stable (with a lower affinity between subunits) and at temperatures above the transition (above 14°) a second state is more stable (with a higher affinity between subunits). Figure 5 is a van't Hoff plot of log K_D against the reciprocal of absolute temperature. From the van't Hoff relationship

$$\frac{d(\ln K)}{d(1/T)} = -\frac{\Delta H^\circ}{R} \tag{15}$$

it is seen that the slope of such a plot yields the molar enthalpy change, ΔH° (R is the universal gas constant). It can be seen (Figure 5) that ΔH° is relatively small at temperatures above and below the transition region (12–14°) but exhibits a sharp variation with temperature through the transition region. From the data at 12 and 14° a value of 72 kcal/mole was calculated for ΔH° over this transition region. The pronounced variation of ΔH° with temperature indicates a reversible change in heat capacity, C_p, for the protein since

$$C_p = \left(\frac{\partial H}{\partial T}\right)_p \tag{16}$$

The thermodynamic functions for subunit association are summarized in Table III. The high values of ΔH° and ΔS° and the sharp change with temperature in association properties strongly suggest a transition between different conformational states of the apoprotein. Similar high values in thermodynamic parameters have been observed for reversible changes in protein conformation of ribonuclease (Hermans and Scheraga, 1961), chymotrypsinogen (Brandts, 1964), and chymotrypsin (Havsteen *et al.*, 1963). Data obtained by Massey *et al.* (1966) have previously indicated the occurrence of conformational changes in D-amino acid oxidase holoenzyme and benzoate complex, but these workers did not find corresponding results for the apoenzyme. In this study we have found a sharp thermal transition for apoenzyme over the range 12–14°, reflected in the apparent equilibrium constant for subunit association. These results obtained with the apoprotein show that the conclusion of Massey *et al.* (1966) is correct inasmuch as the thermal transition is an intrinsic property of the protein rather than the result of protein–FAD binding.

Simultaneous Dimerization and Isomerization. In order to evaluate critically the possibility that the protein may undergo isomerization equilibria between different conformational states in addition to the subunit dimerization, we have carried out an analysis of the type of behavior to be expected for a system undergoing simultaneous dimerization and isomer-

TABLE II: Equilibrium Constants for Apoenyzme at Temperatures between 4 and 20°.

Temp (°C)	K_D (ml/mg)	$10^{-5} K_D$ (M^{-1})[a]	ΔG (kcal/mole)
4.0	3.2 ± 1.2[d]	1.1	−6.4
8.0	3.7	1.3	−6.6
10.0	3.3 ± 0.6[c]	1.2	−6.6
12.0	2.5	0.9	−6.5
14	7.6	2.6	−7.1
16	8.5	3.0	−7.2
18	9.2	3.2	−7.3
20	8.7 ± 3.8[b]	3.1	−7.4

[a] Based on a molecular weight of 35,000 g/mole. [b] Average of 14 determinations. [c] Average of 9 determinations. [d] Average of 8 determinations. Other results represent the average of duplicate determinations.

TABLE III: Thermodynamic Parameters for the Association of D-Amino Acid Oxidase Apoenzyme.

Temp (°C)	ΔG° (kcal/mole)	ΔH° (kcal/mole)	ΔS (eu)	ΔS_u (eu)
4–12	−6.5	0	23	31
12–14		72	230	238
14–20	−7.3	0	25	33

ization. The expected behavior can be determined by considering the following scheme

$$\begin{array}{ccc} 2E_1 & \underset{}{\overset{K_1}{\rightleftharpoons}} & E_2 \\ {\scriptstyle K_3}\downarrow\uparrow & & \downarrow\uparrow{\scriptstyle K_4} \\ 2E_1^* & \underset{K_2}{\rightleftharpoons} & E_2^* \end{array} \qquad (17)$$

where K_1 and K_2 represent the microscopic dimerization constants and K_3 and K_4 represent the microscopic equilibrium constants for isomerization. E_1 and E_1^* represent different conformational states of monomer and E_2 and E_2^*, the corresponding states of dimer.

Analysis of this scheme (see Appendix) revealed that the macroscopic dimerization constant, K_D, determined from the molecular sieve measurements can be resolved into the following combination of microscopic constants

$$K_D = \frac{K_1 + K_2K_3^2}{1 + 2K_3 + K_3^2} \qquad (18)$$

This equation shows that for a given temperature the apparent constant, K_D, calculated from data according to eq 14 is, in fact, a real constant at fixed temperature and is independent of protein concentration. Even though the molecular sieve experiments do not distinguish between different conformational isomers[2] the experimentally determined macroscopic constant, K_D, reflects a contribution of the isomerization reactions through the microscopic constant K_3. This relationship (eq 18) then, provides a rational basis for the existence of a sharp transition in K_D with temperature. The variation of K_1 and K_2 with temperature would not be expected to be abrupt, but the variation in K_3 would be very sharp if the isomerization is a conformational "melting" of the type observed for a number of proteins.

In contrast to the molecular sieve experiments which measure aggregation through changes in molecular size, optical spectra would be expected to provide information regarding the relative proportion of the different conformational states, provided measurements were made in a spectral region sensitive to the conformational difference. For example, in the case of absorbance measurements an apparent isomerization constant K_I may be determined by

$$K_I = \frac{\bar{\epsilon} - \epsilon^*}{\epsilon - \bar{\epsilon}} \qquad (19)$$

where $\bar{\epsilon}$ is the extinction coefficient of the solution at some temperature, ϵ is the extinction coefficient of one isomer, and ϵ^* of the other. In terms of the reaction scheme (eq 17) this apparent "over-all" isomerization constant represents the ratio of total protein in the two conformational states

$$K_I = \frac{(E_1^*) + (E_2^*)}{(E_1) + (E_2)} \qquad (20)$$

When expressed in terms of microscopic equilibrium constants (see Appendix) this apparent equilibrium constant, in sharp contrast to K_D, is seen to be dependent, at constant temperature, upon the total protein concentration (E_T).

$$K_I = \frac{K_3\left\{1 + K_2K_3\left[\dfrac{-(1 + K_3) + \sqrt{(1 + K_3)^2 + 4(K_1 + K_2K_3^2)(E_T)}}{2(1 + K_2K_3^2)}\right]\right\}}{1 + K_1\left[\dfrac{-(1 + K_3) + \sqrt{(1 + K_3)^2 + 4(K_1 + K_2K_3^2)(E_T)}}{2(1 + K_2K_3)}\right]} \qquad (21)$$

Postulation of simultaneous isomerization and subunit dimerization leads to the prediction that conformation-sensitive optical properties should depend upon protein concentration at constant temperature.[3] Several types of spectral measurements were therefore carried out to test this prediction for the D-amino acid oxidase apoprotein. These included concentration difference spectra and optical rotatory dispersion measurements in which the effect of concentration on the measurable parameters was observed.

Optical Studies. Concentration difference spectra were determined according to the method of Fisher and Cross (1965), which allows one to determine the effect of concentration-dependent structural changes upon the environment of chromophoric residues of the protein. The same total quantity of light-absorbing material is exposed to sample and reference light beams but at different concentrations. An example of the type of spectrum obtained is shown in Figure 6, in which the 1-cm sample cell contained D-amino acid oxidase apoenzyme at a concentration of 0.63 mg/ml and the 5-cm cell contained 0.126 mg/ml of apoenzyme. The difference spectrum of the two cells filled with buffer alone has been subtracted. The resultant positive difference spectrum indicated a blue shift on dilution. From the molecular sieve data it is known that a large change in the ratio of dimer to monomer occurs over this concentration range. Under these conditions a broad peak with a maximum at about 267 mμ and two sharper peaks at 279 and 287 mμ are seen. The latter two peaks may be due to tyrosine and the former may be attributable to the exposure of phenylalanine residues to solvent upon dilution (Wetlaufer, 1962).

Optical rotatory dispersion spectra (Figure 7) indicated a concentration dependence of conformation-sensitive optical properties in accordance with the predictions made from the model (eq 17). Figure 7 shows the optical rotatory dispersion spectra obtained with two samples having a tenfold difference in concentration, 0.74 and 0.074 mg/ml. It is clear that there is a change in the specific rotation at 233 mμ with dilution.

[2] It is assumed in the analysis that partition coefficients are identical for the two conformational states of monomer and dimer (see Appendix). This would be the case unless a very drastic change in molecular shape occurred. Such a change would make the observed fit to the data to a dimerization model *most* unlikely. Similarly it is assumed that the optical measurements do not distinguish between E_1 and E_2 or E_1^* and E_2^* but do distinguish between E_1 and E_1^* and between E_2 and E_2^*.

[3] This conclusion is true in general for simultaneous subunit association and isomerization, of which the case analyzed in detail here is the simplest.

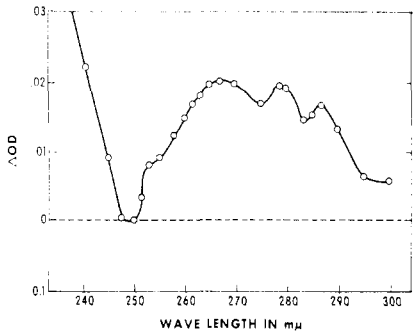

FIGURE 6: Concentration difference spectra of D-amino acid oxidase apoenzyme. A 1-cm sample cell contained 0.63 mg/ml; 5-cm reference cell contained 0.126 mg/ml. Difference spectra of two cells filled with buffer subtracted.

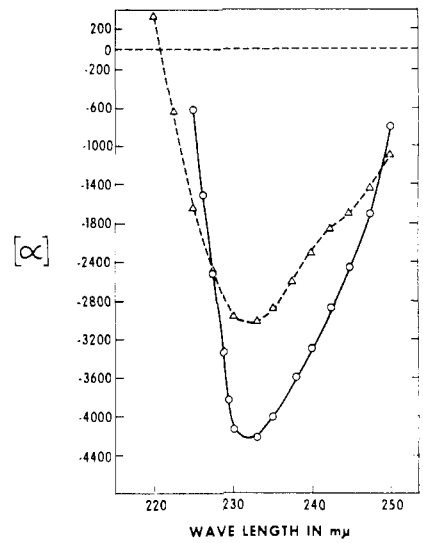

FIGURE 7: Optical rotatory dispersion spectra of D-amino acid oxidase apoenzyme. (———) 0.74-mg/ml sample; (– – – –) 0.074-mg/ml sample.

The more concentrated sample contained 35% monomer as calculated from the equilibrium constant at 20°, 8.8 ml/mg, after correction for the high molecular weight, nondissociating aggregate, and the more dilute sample 72% monomer. Over this concentration range there was observed to be about a 35% change in specific rotation at 233 mμ. Thus, it is indicated that as the protein dissociates there is a decrease in apparent helicity.

Discussion

From the results of this investigation and those of Massey *et al.* (1966), a more complete understanding emerges regarding the role of protein conformation changes and FAD binding in the thermal transition of the enzyme. A striking temperature dependence of the dimerization of D-amino acid oxidase apoenzyme was observed in this work, although not in the studies of these workers (Antonini *et al.*, 1966; Massey *et al.*, 1966). They did see, however, similar effects for the holoenzyme and the benzoate complex. Their only reported investigations on the apoenzyme were at a concentration of 5.22 mg/ml, and it may be that, since light scattering is most sensitive to larger particles, the small changes occurring the amount of low molecular weight components at this high concentration may have been masked by high molecular weight aggregates of denatured protein. Apoenzyme is known from previous studies to be less stable than holoenzyme with respect to heat denaturation (Burton, 1951) and urea treatment (Aki *et al.*, 1964) and the present study confirms the previously noted tendency of apoenzyme to form aggregates of denatured protein in 0.1 M pyrophosphate.

In the present studies it has been found that subunit association of the highly purified apoenzyme is endothermic, proceeding more favorably at higher temperatures than at lower ones, and that there is a marked transition in the equilibrium constant for the dimerization over a temperature range of 12–14°. The midpoint of this transition coincides with that found by Antonini *et al.* (1966) for the holoenzyme and benzoate complex, although the temperature range over which the transition occurs with those two forms was found to be much broader, about 10–20°. In their study of the holoenzyme it was found that lower temperatures appeared to stabilize a form of the protein which shows decreased association and that higher temperature leads to increased association. The same pattern of results were found with the highly purified apoenzyme although the transition region was found to be much more narrow (12–14°). The $\Delta H°$ for the association reaction on either side of the transition temperature was found to be approximately zero, and the $\Delta H°$ over the transition, from 12 to 14°, was 72 kcal/mole. Massey *et al.* (1966) have found a transition temperature of 12–14° in the *holoenzyme* when they investigated the effect of temperature on the sedimentation coefficient, ultraviolet and visible spectra, protein fluorescense, and catalytic activity. They have interpreted this to be due to a temperature-dependent structural transition, and have calculated an enthalpy change for the transition (from 10 to 20°) from the fluorescence data of 78 kcal/mole. This $\Delta H°$ value is consistent with the relatively sharp break in the Arrhenius plot for the catalytic activity. Since the change in catalytic activity reflects the state of active enzyme present in solution, the value of 78 kcal/mole may be regarded as in quite good agreement with the 72 kcal/mole found here for the $\Delta H°$ of transition of apoenzyme.

The relationships indicated by the model developed in this study represent a general approach to the analysis of systems which undergo simultaneous dimerization and isomerization between different conformational states. As predicted by the model concentration-dependent conformation changes are indicated for D-amino acid oxidase apoenzyme in the optical studies. However, due to the presence and formation of aggregates of the unstable apoenzyme the spectral studies cannot yet be quantitatively interpreted. Similar behavior has been noted by Brandts (1964) for chymotrypsinogen, in which irreversible aggregation of the protein to high molecular weight aggregates occurred at high ionic strength. Measurement of a thermodynamically reversible isomerization constant, K_I, as a function of concentration and temperature would permit determination of all four of the microscopic constants of the model and the relative significance of dimerization and isomerization to the observed physical properties of the apoenzyme. When a ligand is added to such a system the analysis becomes much more difficult. Even for the simple model analyzed here there

are then, in addition, at least four possible binding constants, one to each conformer of monomer and dimer. An allosteric effect of the type proposed by Monod *et al.* (1965) might result if the ligand bound preferentially to one of the conformers thereby shifting the subunit isomerization equilibria. On the other hand, an allosteric effect of the type described by Nichol *et al.* (1967) might result if the ligand bound preferentially to one of the polymeric forms. Finally, these effects could be further complicated if the ligand bound preferentially to one of the conformers or polymers and as a consequence of binding changed the equilibrium of association or isomerization.

The fact that the transition temperature found for the highly purified apoenzyme coincided with that found for the catalytically active holoenzyme (Massey *et al.*, 1966) implies that FAD binds equally to each of the two conformers. Any unequal binding would tend to stabilize that conformer to which the ligand was more strongly bound and result in a shift in the transition temperature. If this is indeed the case the increased association observed for the holoenzyme over that of the apoenzyme (Antonini *et al.*, 1966) may result solely from preferential FAD binding to the associated subunits and not from any effect upon the isomerization reactions. This also suggests that FAD binding is not very strongly temperature dependent, since that would shift the transition temperature as well.

Appendix

Parameters of a System Undergoing Simultaneous Dimerization and Isomerization. A system of protein subunits undergoing simultaneous dimerization and conformational isomerization reactions may be represented by the scheme

$$\begin{array}{ccc} 2E_1 & \overset{K_1}{\rightleftharpoons} & E_2 \\ {\scriptstyle K_3}\uparrow\downarrow & & \downarrow\uparrow{\scriptstyle K_4} \\ 2E_1^* & \underset{K_2}{\rightleftharpoons} & E_2^* \end{array} \qquad (1')$$

Different conformers of monomer are denoted E_1 and E_1^* and corresponding conformers of dimer by E_2 and E_2^*. The microscopic dimerization constants are defined by the expressions

$$K_1 = \frac{(E_2)}{(E_1)^2} \qquad (2')$$

$$K_2 = \frac{(E_2^*)}{(E_1^*)^2} \qquad (3')$$

and K_3 and K_4 represent the microscopic isomerization constants.

$$K_3 = \frac{(E_1^*)}{(E_1)} \qquad (4')$$

$$K_4 = \frac{(E_2^*)}{(E_2)} \qquad (5')$$

Only three of the microscopic constants are independent since

$$K_1K_4 = K_2K_3^2 \qquad (6')$$

The apparent dimerization constant, K_D, calculated from experimental data is

$$K_D = \frac{(E_2) + (E_2^*)}{[(E_1) + (E_1^*)]^2} \qquad (7')$$

By substituting for terms (eq 2–6) in the right side of eq 7, K_D can be shown to be a combination of the microscopic constants in the following way

$$\begin{aligned} K_D &= \frac{K_1(E_1)^2 + K_2(E_1^*)^2}{(E_1)^2 + 2(E_1)(E_1^*) + (E_1^*)^2} \\ &= \frac{K_1(E_1)^2 + K_2(E_1)^2K_3^2}{(E_1)^2 + 2K_3(E_1)^2 + K_3^2(E_1)^2} \\ &= \frac{K_1 + K_2K_3^2}{1 + 2K_3 + K_3^2} \end{aligned} \qquad (8')$$

This apparent dimerization constant is the constant determined by the molecular sieve chromatographic experiments provided the conformational isomers for each level of aggregation have indistinguishable partition coefficients. The measured weight-average partition coefficient $\bar{\sigma}_w$ is

$$\bar{\sigma}_w = \frac{(E_1)}{(E_T)}\sigma_1 + \frac{(E_1^*)}{(E_T)}\sigma_1^* + \frac{(E_2)}{(E_T)}\sigma_2 + \frac{(E_2^*)}{(E_T)}\sigma_2^* \qquad (9')$$

If the partition coefficients for the two conformers of the monomer and dimer are identical, *i.e.*, $\sigma_1 = \sigma_1^*$ and $\sigma_2 = \sigma_2^*$ then

$$\bar{\sigma}_w = \frac{(E_1) + (E_1^*)}{(E_T)}\sigma_1 + \frac{(E_2) + (E_2^*)}{(E_T)}\sigma_2$$

and

$$\bar{\sigma}_w = \alpha_T\sigma_1 + (1 - \alpha_T)\sigma_2 \qquad (10')$$

where α_T is equal to the weight fraction of total monomer, $[(E_1) + (E_1)^*]/(E_T)$

$$\alpha_T = \frac{\bar{\sigma}_w - \sigma_2}{\sigma_1 - \sigma_2} \qquad (11')$$

and the experimentally determined dimerization constant is

$$K_D = \frac{(1 - \alpha_T)}{(\alpha_T)^2(E_T)} \qquad (12')$$

A second macroscopic constant by which the system may be characterized is the apparent equilibrium constant representing the ratio of total protein in the two conformational states, as might be determined from optical measurements.

$$K_I = \frac{(E_1^*) + (E_2^*)}{(E_1) + (E_2)} \qquad (13')$$

It is assumed in this analysis that the optical measurements do not distinguish between E_1 and E_2 or E_1^* and E_2^*. This appar-

ent equilibrium constant, K_1, however, unlike K_D is concentration dependent, since

$$K_1 = \frac{K_3(E_1) + K_2(E_1^*)^2}{(E_1) + K_1(E_1)^2} = \frac{K_3(E_1) + K_2K_3^2(E_1)^2}{(E_1) + K_1(E_1)^2} \quad (14')$$

$$K_1 = \frac{K_3[1 + K_2K_3(E_1)]}{[1 + K_1(E_1)]} \quad (15')$$

The total protein concentration (E_T) is equal to the sum of protein concentrations in all forms

$$(E_T) = (E_1) + (E_1^*) + (E_2) + (E_2^*) \quad (16')$$

Substituting expressions 2′–5′ into 16′

$$(E_T) = (E_1) + K_1(E_1)^2 + K_3(E_1)^2 + K_2K_3^2(E_1)^2$$

$$= (E_1)(1 + K_1) + (E_1)^2(K_1 + K_2K_3^2)$$

Thus (E_1) is a quadratic function related to total protein concentration

$$(E_1)^2(K_1 + K_2K_3^2) + (E_1)(1 + K_3) - (E_T) = 0 \quad (17')$$

$$(E_1) = \frac{-(1 + K_3) + \sqrt{(1 + K_3)^2 + 4(K_1 + K_2K_3^2)(E_T)}}{2(K_1 + K_2K_3^2)} \quad (18')$$

Then, by substituting into eq 15′ for (E_1) from eq 18′, the concentration dependence of the macroscopic isomerization constant, K_I, is found to be

$$K_I = \frac{K_3\left\{1 + K_2K_3\left[\frac{-(1 + K_3) + \sqrt{(1 + K_3)^2 + 4(K_1 + K_2K_3^2)(E_T)}}{2(K_1 + K_2K_3^2}\right]\right\}}{1 + K_1\left[\frac{-(1 + K_3) + \sqrt{(1 + K_3)^2 + 4(K_1 + K_2K_3^2)(E_T)}}{2(K_1 + K_2K_3^2)}\right]} \quad (19')$$

If one of the microscopic constants, K_1, K_2, or K_3, is known independently as, for example, by measuring a thermal transition at very low concentrations

$$K_I = \frac{(E_1^*)}{E_1)}$$

K_3 would be approximated since $K_1(E_1)^2$ and $K_2(E_1^*)^2$ would be close to zero

$$K_I = \frac{K_3(E_1)}{(E_1)} = K_3$$

and eq 8′ and 19′ could be used as simultaneous equations in the other variables, K_1 and K_2.

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