A New Method of Preparation of \(\text{d}-\text{Amino Acid Oxidase}\) Apoprotein and a Conformational Change after Its Combination with Flavin Adenine Dinucleotide

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SUMMARY

The apoprotein of \(\text{d}-\text{amino acid oxidase}\) has been prepared by a new method involving dialysis of the holoenzyme against 1 \(\text{M}\) potassium bromide. The apoprotein prepared in this way has been found to have the same properties as that prepared by the classical acid-ammonium sulfate method.

The effect of mixing apoprotein with flavin adenine dinucleotide has been followed by changes in absorbance at 493 \(\text{nm}\), by changes in flavin fluorescence and protein fluorescence, and by measuring catalytic activity. It is found that the reaction proceeds in two stages, a rapid binding of FAD being followed by slow secondary changes which correlate with the appearance of catalytic activity.

Evidence is presented that the slow change is due to a change in protein conformation. Some speculation is made on the significance of this finding to the current theories of protein synthesis.

In the past it has been the practice to prepare the apoprotein of \(\text{d}-\text{amino acid oxidase}\) (\(\text{d}-\text{amino acid: oxygen oxidoreductase}\) (deaminating), EC 1.4.3.3) by the classical method of Warburg and Christian (1) involving the liberation of the prosthetic group at low \(\text{pH}\) (approximately 2.0) in the presence of high concentrations of \((\text{NH}_4)_2\text{SO}_4\) (approximately 0.5 saturation). This procedure was in fact the basis of the classical purification of the enzyme as the apoprotein by Negelein and Brömel (2). Since the isolation of the holoenzyme as a pure crystalline enzyme by Massey, Palmer, and Bennett (3) or modifications of this procedure (4, 5), the pure apoprotein was readily available by the classical acid-(\(\text{NH}_4\))\(_2\text{SO}_4\) resolution. This paper describes the preparation of the apoprotein, a comparison of its properties with that of the acid-(\(\text{NH}_4\))\(_2\text{SO}_4\) apoprotein, and the evidence for a protein conformation change accompanying re-formation of the active holoenzyme.

METHODS

\(\text{d}-\text{Amino acid oxidase}\) holoenzyme was prepared by a slight modification (4) of the method originally described (3). Benzoate, used to stabilize the holoenzyme during purification, was removed as previously described (4). Apoprotein was prepared either by the method of Strittmatter (6) or by dialysis against KBr. In the latter procedure benzoate-free holoenzyme was dialyzed at 4\(^\circ\) against a solution of 0.1 \(\text{M}\) pyrophosphate, \(\text{pH}\) 8.5, containing \(3 \times 10^{-3} \text{M}\) EDTA and 1 \(\text{M}\) KBr. The dialysate was changed three to four times over a 2-day period until the yellow color of the holoenzyme had disappeared. The dialyzing medium was then changed to 0.1 \(\text{M}\) pyrophosphate, \(\text{pH}\) 8.5, to remove KBr; dialysis was continued for 1 to 2 days with several changes of dialyzing medium to ensure complete removal of KBr. The resulting apoprotein was stable for several weeks at 0\(^\circ\) and for at least several months if stored at -20\(^\circ\). Measurements of enzyme activity were made manometrically as described previously (3). Spectra were recorded with a Cary model 14 spectrophotometer equipped with thermostated cell holders. Fluorescence measurements were made with an Aminco-Bowman spectrophotometer with the use of slits of \(\frac{1}{2}, \frac{1}{4}, \frac{1}{8}, \frac{1}{16}, \frac{1}{32}\), and \(\frac{1}{64}\) inch between the light source and photomultiplier tube. This instrument was also equipped with a thermostated cell holder.

For flavin fluorescence experiments, chromatographically pure FAD made by the procedure of Massey and Swoboda (9)
One of the disadvantages of the acid-(NH₄)₂SO₄ resolution procedure is that the yield of apoprotein is somewhat variable; considerable denaturation is often obtained, depending on the pH and temperature at which the separation of precipitated apoprotein and acid supernatant solution is performed. Yields with this procedure are typically in the 60 to 80% range, although values on either side of this range are sometimes obtained. In contrast, the KBr-dialysis method gives apoprotein reproducibly in almost 100% yield. While the yields are thus different, the apoprotein appears to be the same whether made by the acid-(NH₄)₂SO₄ or by the KBr dialysis method. Table I summarizes some of the analytical data. Furthermore, within experimental error, the FAD titration curve is the same with apoprotein from both methods, and the full activity of the native enzyme is regained as shown in Fig. 1. Similarly, the protein fluorescence spectra are quantitatively the same, as shown in Fig. 2. This should be a very sensitive technique for detecting differences in protein structure, as the fluorescence yield would be expected to depend largely on tryptophan-solvent interaction. Thus, in a variety of tests, no difference was found between apoprotein made by the two methods; in all the subsequent experiments to be described, apoprotein prepared by the KBr method was used.

Spectral Differences between FAD and D-Amino Acid Oxidase and Use to Follow Apoprotein-FAD Recombination—One of the characteristic features of flavoproteins in general is that they show considerable differences in spectrum not only compared with one another, but also compared with the free prosthetic group. This is illustrated in Fig. 3 for D-amino acid oxidase. This property may be used conveniently to monitor the recombination of FAD with apoprotein, as the latter has no absorbance in the visible region. In all the experiments that follow in which this property has been used, the increase in absorbance at 493 mμ has been followed. A blank cuvette containing the same concentration of FAD as added to the apoprotein was used as a spectral blank. When apoprotein-FAD interaction was followed in this way, it was found that the reaction was surprisingly slow, particularly at low temperatures. This phenomenon is shown in Fig. 4 for four temperatures. For any temperature, after a short initial lag period, the absorbance changes follow first order kinetics. The slowness of the changes and the obtaining of apparent first order kinetics were so striking as to cause skepticism whether these changes could be due simply to the expected equilibrium, apoprotein + FAD $\leftrightarrow$ holoenzyme. Instead it seemed possible that what we were observing was a two-step process, a rapid binding of FAD to apoprotein (not accompanied by spectral change), followed by a secondary change in the structure of the apoenzyme-FAD complex which gave rise to the spectral changes. Such a scheme could account for the initial lag, the apparent first order kinetics, and the slowness of the reaction. That this explanation is correct is strongly suggested by the results shown in Fig. 5, which show the first order plots for a series of experiments at 15.2°, where the FAD concentration was kept constant and the apoprotein concentration varied over a 5-fold range. In all cases the same rate constant, 0.19 min⁻¹, was obtained. This is entirely in keeping with the scheme proposed and very difficult to reconcile with

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**RESULTS**

One of the disadvantages of the acid-(NH₄)₂SO₄ resolution procedure is that the yield of apoprotein is somewhat variable; considerable denaturation is often obtained, depending on the pH and temperature at which the separation of precipitated apoprotein and acid supernatant solution is performed. Yields with this procedure are typically in the 60 to 80% range, although values on either side of this range are sometimes obtained. In contrast, the KBr-dialysis method gives apoprotein reproducibly in almost 100% yield. While the yields are thus different, the apoprotein appears to be the same whether made by the acid-(NH₄)₂SO₄ or by the KBr dialysis method. Table I summarizes some of the analytical data. Furthermore, within experimental error, the FAD titration curve is the same with apoprotein from both methods, and the full activity of the native enzyme is regained as shown in Fig. 1. Similarly, the protein fluorescence spectra are quantitatively the same, as shown in Fig. 2. This should be a very sensitive technique for detecting differences in protein structure, as the fluorescence yield would be expected to depend largely on tryptophan-solvent interaction. Thus, in a variety of tests, no difference was found between apoprotein made by the two methods; in all the subsequent experiments to be described, apoprotein prepared by the KBr method was used.

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**Table I**

Comparison of D-amino acid oxidase apoprotein prepared by acid-(NH₄)₂SO₄ and KBr resolution

<table>
<thead>
<tr>
<th>Method</th>
<th>Yield</th>
<th>Activity</th>
<th>Eₘ₉ for 10% solution</th>
<th>kₘ⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-(NH₄)₂SO₄</td>
<td>60-80</td>
<td>140-150</td>
<td>15.4</td>
<td>0.034</td>
</tr>
<tr>
<td>KBr dialysis</td>
<td>93-100</td>
<td>140-150</td>
<td>15.4</td>
<td>0.035</td>
</tr>
</tbody>
</table>

* Under the assay conditions described in the legend of Fig. 1.

* First order rate constant of the change in absorbance at 493 mμ on addition of excess FAD to apoprotein. See later figures for details.

* At pH 1.8 and 0.5 saturation with (NH₄)₂SO₄ at 0°.
simple equilibrium. The effect of temperature on the rate of
holoenzyme formation is shown in the form of an Arrhenius
plot in Fig. 6; the $\Delta H$ of the reaction is 20,200 cal per mole.\(^1\)

\(^1\)The linear Arrhenius plot of Fig. 6 should be compared with
the curves with sharp breaks around 12-14$^\circ$ obtained with the
holoenzyme when a variety of properties were studied as a function
of temperature (10, 11). As indicated in the papers cited, no
temperature-induced conformational change is found with the
apoprotein; similarly, the results of Fig. 6 would indicate the

Quenching of FAD Fluorescence on Addition of Apoprotein—
Very strong support for the multistep association hypothesis
comes from studies of the fluorescence changes on addition of
apoprotein to FAD. In distinction to the changes in absorbance
absence of such a temperature-dependent change in configuration
also in the enzyme-FAD complex. Thus the existence of the
temperature-sensitive conformational change is restricted to the
active holoenzyme (see “Discussion” for differentiation between
enzyme-FAD complex and holoenzyme).

Fig. 2. Comparison of the fluorescence excitation and emission spectra of apoprotein made by two different methods. Conditions:
0.1 M pyrophosphate, pH 8.5; 20$^\circ$; protein concentration, 0.14 mg per ml. O, apoprotein made by KBr dialysis; X, apoprotein made by
the acid-(NH$_4$)$_2$SO$_4$ method. The activation and the emission spectra are uncorrected for photomultiplier response.

Fig. 3. Comparison of absorption spectra of FAD (Curve 1) and D-amino acid oxidase holoenzyme (Curve 2) in 0.1 M pyrophosphate,
pH 8.5, at 17$^\circ$. In both cases experimental spectra were run at a concentration of FAD of $6.9 \times 10^{-6}$ M.
Protein Conformation Changes Associated with d-Amino Acid Oxidase

Fig. 4. Effect of temperature on the change in absorbance at 493 nm on mixing $5.25 \times 10^{-4} \text{ M}$ apoprotein (molarity based on FAD-combining weight of 45,500) with $2.1 \times 10^{-4} \text{ M}$ FAD in 0.1 M pyrophosphate, pH 8.5.

Fig. 5. Effect of apoprotein concentration on the first order rate constant of change in absorbance at 493 nm on mixing apoprotein and FAD. All experiments were carried out in 0.1 M pyrophosphate, pH 8.5, at 15.9°C, with a concentration of FAD of $2.1 \times 10^{-4} \text{ M}$. Curve 1, $1.57 \times 10^{-4} \text{ M}$ apoprotein; 2, $1.05 \times 10^{-4} \text{ M}$ apoprotein; 3, $5.25 \times 10^{-4} \text{ M}$ apoprotein; 4, $3.14 \times 10^{-4} \text{ M}$ apoprotein. The apoprotein concentration is based on the FAD-combining weight of 45,500.

The results of such an experiment carried out at 15.9°C are shown in Fig. 7. When a first order plot of these changes is made, the results shown in Fig. 8 are obtained. The slow secondary change has a rate constant of 0.182 min$^{-1}$, very similar to that described above, a large and rapid decrease in fluorescence was followed by a much slower secondary decrease in fluorescence.
observed at a similar temperature when the change in absorbance at 493 nm is followed. This rate constant is again independent of the concentration of the apoprotein; however, the time taken for the rapid fluorescence change to occur is quite dependent on concentration. If we assume that the results are due to consecutive reactions,

$$E + FAD \rightarrow EFAD \rightarrow holoenzyme$$

the pseudo-first order rate constant for the fast phase may be calculated approximately by subtracting the contribution of the slow phase and replotting the differences. This is shown in Fig. 8 as the broken line, yielding a pseudo-first order rate constant of 2.5 min⁻¹. As the apoprotein concentration was 1.89 × 10⁻⁵ M, this is equivalent to a second order rate constant of 1.3 × 10⁵ M⁻¹ min⁻¹.

![Fig. 7. Changes in flavin fluorescence on mixing 1.76 × 10⁻⁴ M chromatographically pure FAD with 1.89 × 10⁻⁵ M apoprotein in 0.1 M pyrophosphate, pH 8.5, at 15.9°. Flavin fluorescence was isolated by excitation at 470 nm and by following the emission at 530 nm.](image)

![Fig. 8. First order analysis of the results shown in Fig. 7. O, primary plot; ----, results obtained for the fast phase after subtracting the contribution of the slow phase.](image)

**Fig. 7.** Changes in flavin fluorescence on mixing 1.76 × 10⁻⁴ M chromatographically pure FAD with 1.89 × 10⁻⁵ M apoprotein in 0.1 M pyrophosphate, pH 8.5, at 15.9°. Flavin fluorescence was isolated by excitation at 470 nm and by following the emission at 530 nm.

**Fig. 8.** First order analysis of the results shown in Fig. 7. O, primary plot; ----, results obtained for the fast phase after subtracting the contribution of the slow phase.

![Fig. 9. Changes in the protein fluorescence (excitation, 305 nm; emission, 340 nm) on mixing 8 × 10⁻⁴ M apoprotein with 1 × 10⁻⁵ M FAD in 0.1 M pyrophosphate, pH 8.5, at 15.9°. The initial fluorescence is corrected for the small quenching due to the absorbance of the FAD added. This was determined in one of two ways: by adding FAD to bovine serum albumin or by adding FMN to apoprotein. In both instances a quenching of 12% was observed.](image)

**Fig. 9.** Changes in the protein fluorescence (excitation, 305 nm; emission, 340 nm) on mixing 8 × 10⁻⁴ M apoprotein with 1 × 10⁻⁵ M FAD in 0.1 M pyrophosphate, pH 8.5, at 15.9°. The initial fluorescence is corrected for the small quenching due to the absorbance of the FAD added. This was determined in one of two ways: by adding FAD to bovine serum albumin or by adding FMN to apoprotein. In both instances a quenching of 12% was observed.

**Fig. 10.** First order plot of the results shown in Fig. 9.

**Quenching of Protein Fluorescence of Apoprotein or Combination with FAD—**When the fluorescence of protein was used to monitor the effect of recombination of FAD and apoprotein, very similar results were obtained as with flavin fluorescence, except that only a small decrease in fluorescence occurred rapidly, followed by a large slow change. The results of such an experiment carried out at 15.9° are shown in Fig. 9, and the first order plot is given in Fig. 10. Again the slow phase has a rate constant similar to that observed in the increase in absorbance at 493 nm (0.217 min⁻¹), and again this rate constant is independent of apoprotein concentration. The time taken for the fast phase to be complete is too small to permit accurate calculations, but it is...
Fig. 11. Oxygen uptake measured in Warburg manometers when FAD and apoprotein were mixed at zero time in 0.04 M pyrophosphate, pH 8.5, at 1.4°C, in the presence of D-alanine as substrate. The experiment was performed in quadruplicate; the oxygen uptake shown is the average of that obtained in the four flasks. Each flask contained, in a reaction volume of 2.5 ml, sodium pyrophosphate, pH 8.5, 100 μmoles; EDTA, 6 μmoles; D,L-alanine, 112 μmoles; FAD, 100 μmoles; crystalline horse liver catalase, 10 μg; crystalline bovine serum albumin, 2 mg; and D-amino acid oxidase apoprotein, 225 μg. The center well contained 0.2 ml of 25% (w/v) KOH. The reaction was initiated by tipping apoprotein in from the side arm, the FAD being in the main compartment.

Fig. 12. Analysis of the results of Fig. 11. Enzyme activity at the given times after the addition of FAD to apoprotein (expressed as microliters of O₂ uptake in a 10-min period) was calculated by drawing tangents to the curve shown in Fig. 11. The change in activity with time is described by a first order reaction with a rate constant of 0.044 min⁻¹, quite consistent with the second order rate constant of 1.3 × 10⁴ M⁻¹ min⁻¹. The change in ultraviolet fluorescence on combination with FAD is very large, and it was clearly of interest to determine how many tryptophan residues might be involved. By quantitative comparison of the ultraviolet fluorescence of apoprotein and free tryptophan and with the use of the perhaps unwarranted assumption that the quantum yield of tryptophan fluorescence is the same in the protein as with free tryptophan, it can be calculated readily that the observed fluorescence of the apoenzyme is equivalent to 6.1 moles of tryptophan per mole of apoprotein. Independent analysis of tryptophan by two different methods (12, 13) yield a value of 10.7 to 12 moles of tryptophan. Thus in the apoprotein it can be estimated that roughly one-half the tryptophan residues are probably exposed to solvent interaction. On combination of the apoprotein with FAD, this value decreases by a further factor of 2, indicating that now only 3 tryptophan residues are exposed to the solvent. This conclusion is of course highly equivocal, since the possibilities exist that

### Table II

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Attainement of equilibrium, $E^+ \rightarrow FAD \rightarrow E^+ FAD (s)$</th>
<th>Attainement of equilibrium, $E^+ FAD \rightarrow E^+, FAD (s)$</th>
<th>Method of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4°C</td>
<td>$m^{-1} \text{ min}^{-1}$</td>
<td>0.035</td>
<td>Spectrophotometric, $\Delta A_{405}$</td>
</tr>
<tr>
<td>15.2</td>
<td>$1.3 \times 10^4$</td>
<td>0.10†</td>
<td>Spectrophotometric, $\Delta A_{405}$</td>
</tr>
<tr>
<td>15.9</td>
<td>$1.3 \times 10^4$</td>
<td>0.182</td>
<td>Changes in flavin fluorescence</td>
</tr>
<tr>
<td>15.9</td>
<td>0.217</td>
<td>Changes in protein fluorescence</td>
<td></td>
</tr>
</tbody>
</table>

* Determined with D-alanine as substrate.
† Determined with D-isoleucine as substrate.
‡ Constant with four different concentrations of apoprotein.
all of the protein tryptophan residues have approximately half the quantum yield of free tryptophan or, alternatively, that 2 or 3 tryptophan residues have very high quantum yields, and the remainder, very low quantum yields. With these admittedly serious reservations, the results are still interesting, as they are at least consistent with the hypothesis to be developed (see "Discussion") that the slow changes in properties observed after FAD binding are due to a protein conformational change.

**Discussion**

The results of this study show clearly that the re-formation of D-amino acid oxidase holoenzyme from FAD and apoprotein is not a simple two-stage process, a comparatively rapid binding of FAD being followed by slower secondary changes. Thus instead of the expected simple relationship of Equation 1, we must postulate the more complex relationship of Equation 2.

\[
E + \text{FAD} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{holoenzyme} \tag{1}
\]

\[
E + \text{FAD} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{BFAD} \overset{k_2}{\rightarrow} \text{holoenzyme} \tag{2}
\]

The evidence for the scheme shown in Equation 2 has been described under "Results" and is summarized in Table II. The rapid attainment of the first equilibrium is characterized by no apparent change in absorbance at 493 m\(\mu\), by a large quenching of flavin fluorescence, by a small decrease in protein fluorescence, and by no apparent catalytic activity. The slow attainment of the second equilibrium is characterized by a large change in absorbance at 493 m\(\mu\), by a small further decrease in flavin fluorescence, by a large quenching of protein fluorescence, and by the attainment of catalytic activity. All of these changes are well correlated on a kinetic basis. The secondary slow phase in these characteristics after FAD binding would appear to us to be associated with a change in the conformation of the protein, this conformational change being required for enzyme activity. The slow change is due to protein conformational changes rest mainly on the large secondary change of protein fluorescence, which must be due mainly to tryptophan residues. As can be seen readily, there was a pronounced lag in the uptake of O\(\_2\), and a constant rate of O\(\_2\) uptake was not obtained until about 80 min after apoprotein and FAD were mixed. By drawing tangents at various times to the curve of Fig. 11, the increase in catalytic velocity with time of incubation can be determined. The results shown in Fig. 11 were obtained.

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