Evidence for Conformational Changes in L-Amino Acid Oxidase Associated with Reversible Inactivation*

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ABSTRACT: The active and inactive forms of L-amino acid oxidase were compared by spectrophotometry, optical rotatory dispersion, and immunochemical techniques in an effort to detect differences in conformation. Upon inactivation, the visible spectrum of the enzyme shifted toward shorter wavelengths and the absorbancy at 275 mμ decreased. Optical rotatory dispersion measurements of the active form of the enzyme revealed several Cotton effects in the regions of the flavin–adenine dinucleotide absorption maxima. The inactive form of the enzyme was considerably more levorotatory than the active form in the visible range, and the Cotton effects were less pronounced. Both forms exhibited a trough at 231 mμ. All the changes in spectrum and optical rotatory dispersion were completely reversed on reactivation.

The active and inactive enzymes reacted in the same way with rabbit anti-L-amino acid oxidase as determined by complement fixation and Ouchterlony double diffusion tests. The active enzyme reacted with the antibody to yield a catalytically active precipitate. The two forms of the enzyme exhibited identical sedimentation coefficients and electrophoretic mobilities. It is concluded that inactivation and reactivation are associated with conformational changes at the active center of the enzyme and changes in the mode of binding of flavin–adenine dinucleotide to the protein. Inactivation does not involve aggregation, dissociation into subunits, resolution of the enzyme, or changes in the antibody binding site. These results are discussed in terms of the hypothesis presented earlier that the two flavins are part of the same active center.

The L-amino acid oxidases of rattlesnake (*Crotalus adamanteus*) and moccasin (*Agkistrodon piscivorus piscivorus*) venoms have been shown to have a molecular weight of about 130,000 and to contain 2 moles of tightly bound FAD[1]/mole of enzyme (Wellner and Meister, 1960a; Meister and Wellner, 1963). The enzyme from rattlesnake venom occurs in three electrophoretically separable forms, but only one form of the enzyme from moccasin venom has thus far been found (Singer and Kearney, 1950). Although the four enzymes exhibit different solubilities and electrophoretic mobilities, they have identical absorption spectra, enzymatic activity, specificity, and kinetics. Singer and Kearney (1951) reported that the enzyme from moccasin venom is capable of undergoing an unusual type of reversible inactivation. The rates of inactivation and reactivation were found to be strongly temperature dependent, and the equilibrium position between active and inactive enzyme was dependent on pH and on the concentration of Cl^- or other monovalent anions. We have found that the pure enzyme from rattlesnake venom behaves similarly. In view of the suggestions made previously (Wellner and Meister, 1961) concerning the mechanism of action of L-amino acid oxidase, it was of interest to investigate further the inactivation phenomenon. On the basis of a kinetic analysis of the enzymatic reaction and other data it was concluded that each of the two flavins on the enzyme accepts one hydrogen atom from the amino acid substrate, yielding an intermediate enzyme form in which both flavins are half reduced. This intermediate may be reoxidized by oxygen or reduced to the fully reduced form by a second amino acid molecule. It was postulated that the fully reduced enzyme could be reoxidized by oxygen either to the enzyme form containing two half-reduced flavins or to an enzyme form containing one oxidized and one fully reduced flavin. The latter form would not be expected to be reduced by substrate if the substrate reacts by donating one hydrogen to each flavin. Spectrophotometric and kinetic data consistent with the existence of each of the intermediates described above have been obtained (Beinert, 1957; Wellner and Meister, 1960b; Wellner and Meister, 1961). Recently, Gibson (1965), using a stopped-flow apparatus, has obtained additional evidence supporting the above mechanism. If this mechanism is correct, it may be expected that a conformational change in the protein which altered the distance between the flavins or their relative orientation on the enzyme would result in loss of activity. In the present work a comparison between the active and inactive enzymes from rattlesnake venom has been made by spectrophotometry, spectropolarimetry, and im-

* From the Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts. *Received December 23, 1965.* Supported in part by a grant AM-08462) from the National Institutes of Health, U. S. Public Health Service. Some of these findings were presented at the Symposium on Flavins and Flavoproteins, Amsterdam, The Netherlands, June 10–15, 1965 (Wellner, 1965).

† Recipient of a Lederle Medical Faculty Award.

[1] Abbreviations used are: FAD, flavin–adenine dinucleotide; FMN, flavin mononucleotide; C′, complement.

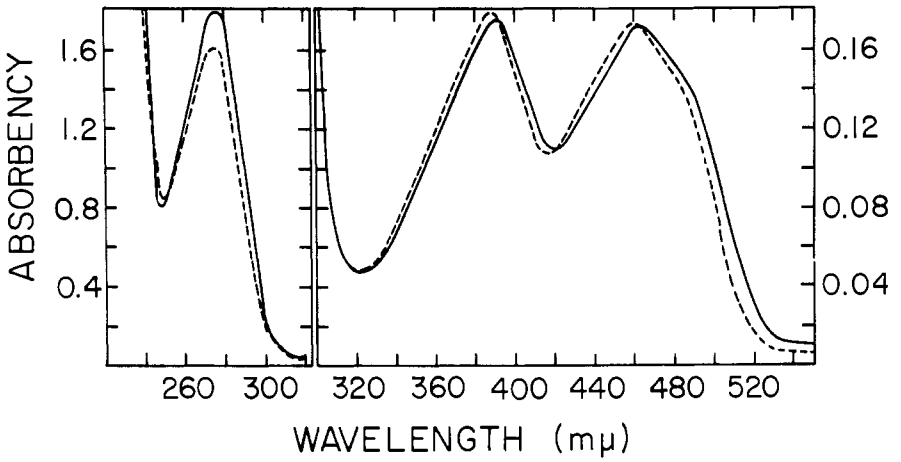

FIGURE 1: Absorption spectra of active (———) and inactivated (-------) L-amino acid oxidase. Protein concentration: 1 mg/ml, in 0.1 M Tris–HCl buffer, pH 7.2; light path, 1 cm, 25°.

munochemical techniques. The data suggest that inactivation is associated with a conformational change at the active center of the enzyme and that FAD is bound in a different manner in the active and inactive enzymes.

Materials

Lyophilized venom of *C. adamanteus* was obtained from Ross Allen's Reptile Institute, Silver Springs, Fla. Crystalline beef liver catalase, FAD (96% pure), and FMN were obtained from the Sigma Chemical Co. Amino acids and monosaccharides were obtained from Calbiochem. The reagents used in the immunochemical studies were generously given by Dr. David Stollar.

Methods

L-Amino acid oxidase was purified and crystallized as described previously (Wellner and Meister, 1960a). All experiments were carried out with twice crystallized enzyme. Determinations of enzyme activity were carried out by a modification of the method described previously (Wellner and Meister, 1960a), which makes use of the high absorbancy of the borate–enolphenylpyruvate complex (Knox and Pitt, 1957). Reaction mixtures containing enzyme (2–30 units as defined previously, Wellner and Meister, 1960a), catalase (60 units), Tris–HCl buffer, pH 7.5 at 38° (80 μmoles), and L-phenylalanine (4 μmoles, added last) in a final volume of 0.8 ml were incubated in 18 × 150 mm test tubes with vigorous shaking (200 excursions/min) at 38° for exactly 15 min. The reaction was stopped by adding 0.2 ml of 25% (w/v) trichloroacetic acid. The reaction mixtures were transferred to conical centrifuge tubes (not suitable for the enzymatic reaction because of inadequate equilibration with the atmosphere) and centrifuged. A 0.5-ml aliquot of the supernatant was then added to 2.5 ml of a solution containing 1 M boric acid and 1 M sodium arsenate (adjusted to pH 6.5 with HCl) and mixed well. After standing at least 30 min at room temperature (22–26°) the absorbency at 300 mμ was measured. When pure L-amino acid oxidase was assayed, no deproteinization was necessary and, therefore, 5 ml of the borate–arsenate buffer was added directly to the reaction tubes. Under these conditions, 1 unit of enzyme gave an optical density of 0.03. A specific activity of 7000 (Wellner and Meister, 1960a) is equivalent to 12.5 units/μg.

For the inactivation experiments, a crystalline enzyme suspension was dissolved in 0.1 M sodium phosphate buffer, pH 7.5, or an enzyme solution was passed through a column of Sephadex G-25 which had been previously equilibrated with this buffer. The enzyme was inactivated by incubation at 38° for 60 min. Under these conditions, <5% of the initial activity remained. The enzyme was reactivated by bringing the solution to pH 5.0 by adding acetic acid and then incubating for 60 min at 38°; 80–100% of the original activity was thus recovered. Control solutions of enzyme were treated similarly except that they were always maintained at 0°. The enzyme solutions were exhaustively dialyzed at 4° *vs.* 0.1 M Tris–HCl buffer, pH 7.2, prior to making the physical measurements.

Protein concentrations were determined by dry weight measurements or refractometrically, using the Rayleigh interference optical system of the Spinco Model H electrophoresis apparatus. Absorption spectra were recorded with a Cary Model 14 spectrophotometer.

Optical rotatory dispersion measurements were made with a Cary Model 60 recording spectropolarimeter. The following precautions were observed in order to obtain reproducible results. The instrument was allowed to warm up for at least 1 hr before use. Cells were always placed in the same position in the holder. Each curve was traced at least two or three times. A base line, using pure solvent, was taken before and after each series of measurements. The results are expressed as specific rotations, $[\alpha]_\lambda^t$, or as molar rotations, $[M]_\lambda^t$.

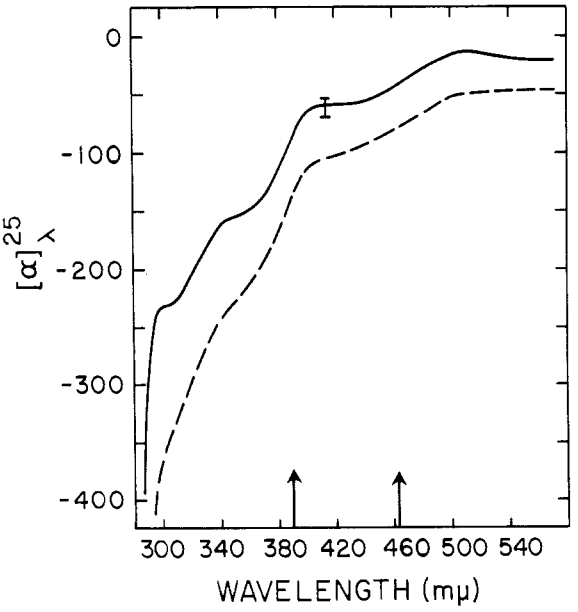

FIGURE 2: Visible and near-ultraviolet optical rotatory dispersion of active (———) and inactivated (------) L-amino acid oxidase in 0.1 M Tris–HCl buffer, pH 7.2. The vertical line indicates the estimated experimental uncertainty. The arrows mark the wavelengths of maximal absorption of the active enzyme.

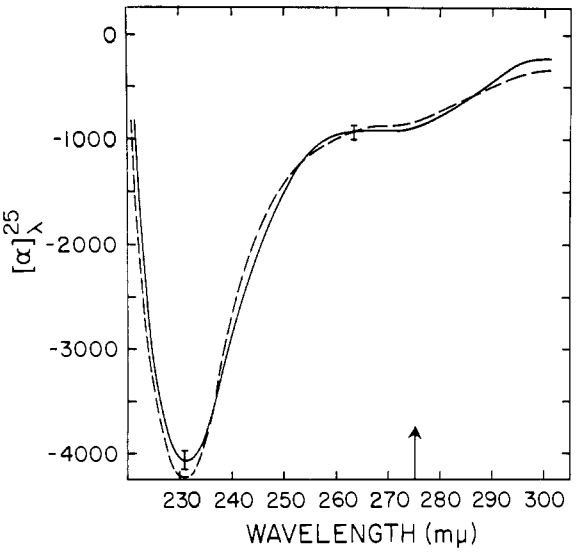

FIGURE 3: Ultraviolet optical rotatory dispersion of active (———) and inactivated (-------) L-amino acid oxidase in 0.1 M Tris–HCl buffer, pH 7.2. The vertical lines indicate the estimated experimental uncertainty. The arrow marks the wavelength of maximal absorption of the enzyme.

Antibody to L-amino acid oxidase was obtained by injecting 5 mg of the crystalline enzyme suspended in complete Freund's adjuvant in the four foot pads of a rabbit. Eleven weeks later an intramuscular injection of 5 mg of enzyme was given. The animal was bled 1 week after the second injection, and the serum was separated and stored at −15°.

Ouchterlony double diffusion tests were done in 1% Difco Bacto agar gel made in 0.1 M Tris–HCl, pH 7.2. Diffusion was allowed to proceed for 48 hr at 4°. (For a review of this technique, see Ouchterlony, 1962.)

Quantitative microcomplement fixation was performed essentially as described by Wasserman and Levine (1961). All dilutions were made in isotonic Tris–HCl buffer, pH 7.4, containing 5×10^{-4} M $MgCl_2$, 1.5×10^{-4} M $CaCl_2$, and 0.1% bovine serum albumin. The antibody, the antigen, and complement (C′) in a final volume of 6 ml were allowed to react for 14 hr at 4°. The amount of free C′ remaining was then determined by its ability to lyse sensitized erythrocytes. The best results were obtained with serum diluted 39,000 times in the final reaction mixture, and, under these conditions, the maximal response was obtained with 30–40 mμg of L-amino acid oxidase. Thus, complement fixation represents a more sensitive method of detecting the enzyme than measurements of enzymatic activity as described above. Complement fixation experiments with reduced enzyme were unsuccessful when carried out under nitrogen, because traces of H_2O_2 produced enzymatically oxidized hemoglobin to methemoglobin, even in the presence of added catalase. This difficulty was overcome by performing the anaerobic experiments in an atmosphere of carbon monoxide.

Results

In their study of the reversible inactivation of L-amino acid oxidase, Singer and Kearney (1951) observed no appreciable change in the sedimentation coefficient or the electrophoretic mobility of the enzyme. We have confirmed these findings. Thus, when the active and inactive enzyme were centrifuged simultaneously in the same rotor, a difference of only 1% in the sedimentation coefficient (which is considered to be within experimental error) was observed. The observed inactivation, therefore, does not involve aggregation or dissociation of the enzyme into subunits.

A comparison of the spectra of the active and the inactive enzyme, shown in Figure 1, indicates that the FAD is not released from the enzyme during inactivation. This conclusion is supported by the finding that, even after dialysis of the inactive enzyme, addition of FAD is not required to obtain reactivation.

As shown in Figure 1, the spectrum of the enzyme above 320 mμ is shifted toward shorter wavelengths on inactivation. Thus, the maxima at 390 and 462 mμ are shifted to 387 and 458 mμ, respectively. Figure 1 also shows that the absorption peak at 275 mμ is lower by about 10% in the inactive enzyme, but there is no shift in wavelength of maximum absorption. When the inactive enzyme was reactivated, the spectrum returned to that of the active enzyme.

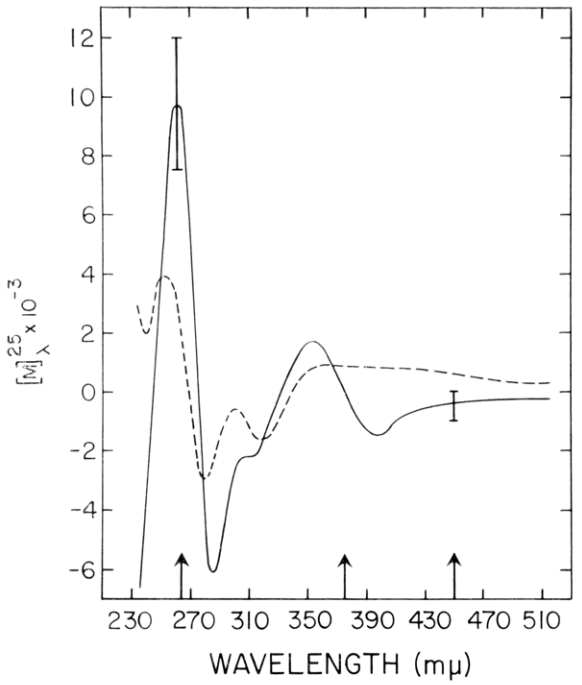

FIGURE 4: Optical rotatory dispersion of FAD (———) and FMN (-----) in 0.05 M sodium phosphate buffer, pH 7.0. The vertical lines indicate the estimated experimental uncertainty. The arrows mark the wavelengths of maximal absorption of FAD.

Optical Rotatory Dispersion. Figures 2 and 3 give the results of optical rotatory dispersion measurements of the active and inactive forms of L-amino acid oxidase. The curve obtained with the active enzyme is characterized by a trough at 231 mμ ($[\alpha]_{231}^{25}$ −4100°) and a relatively complex structure with at least nine points of inflection above 231 mμ. The inactive enzyme is significantly more levorotatory than the active enzyme above 295 mμ, and exhibits a more plain, although still anomalous, dispersion curve. Thus, the bends at 300 and 360 mμ, which are very pronounced in the curve of the active enzyme, are much less apparent in the inactive enzyme. The inactive enzyme exhibits a trough at 231 mμ and is slightly more levorotatory than the active enzyme at this wavelength. When the enzyme was reactivated, an optical rotatory dispersion curve identical with that of the original active enzyme was obtained.

In order to determine to what extent FAD itself contributes to the rotation of the enzyme, optical rotatory dispersion curves of FAD as well as FMN were determined. These are shown in Figure 4. Since both FAD and FMN have relatively small rotations and large absorbencies, it was difficult to obtain accurate results because of the low concentrations which had to be used. Nevertheless a number of Cotton effects are clearly visible in the curves of both coenzymes. FAD has a negative Cotton effect with an inflection point at 375 mμ which is not seen with FMN. In addition, FAD has a large negative Cotton effect with an inflection point at 275 mμ which appears to correspond to a smaller negative Cotton effect with an inflection point at 270 mμ in FMN. FMN also has a small negative Cotton effect with an inflection point at 310 mμ which is barely discernible in FAD, perhaps because it is hidden by the two much larger ones on each side.[2]

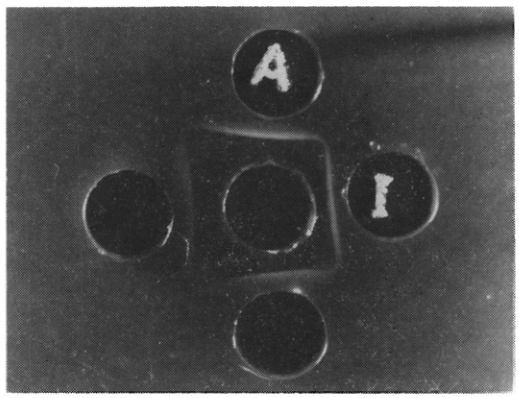

FIGURE 5: Ouchterlony double diffusion test. The center well contained 0.05 ml of immune rabbit serum. Well A contained active, and well I inactive, L-amino acid oxidase (0.1 mg/ml, 0.05 ml). The proteins were allowed to diffuse for 48 hr at 4°.

It may be calculated that free FAD, at a concentration equivalent to that of the bound FAD in the enzyme solutions used for the rotation measurements, would contribute at all wavelengths <3% of the rotation observed with the enzyme.

Immunochemical Studies. In order to obtain more information on possible conformational changes occurring in the enzyme during inactivation, antibody was obtained by injecting the crystalline enzyme in a rabbit. Although the pure enzyme gave a single precipitation line in the Ouchterlony double diffusion test, rattlesnake venom exhibited at least four lines. This shows that there are other substances in the venom which have antigenic determinants similar to those of L-amino acid oxidase toward which the antibody was directed. When the active and the inactive enzyme were placed in adjacent wells in the agar, their precipitation lines coalesced completely, as shown in Figure 5. This result would be expected if the two forms of the enzyme reacted with the same antibody and their antibody combining sites were identical.

A more sensitive test of the interaction of the antibody with its antigen is the complement fixation reaction. In this reaction, in agreement with the results of the Ouchterlony test, identical curves were obtained with the active, the inactive, and the reactivated enzymes

[2] While this paper was in preparation, a preliminary communication by Gascoigne and Radda (1965) on the optical rotatory dispersion of flavin nucleotides has appeared. An optical rotatory dispersion curve for FAD similar to that presented here (Figure 4) has been obtained by Dr. Robert T. Simpson and Dr. Bert L. Vallee (personal communication).

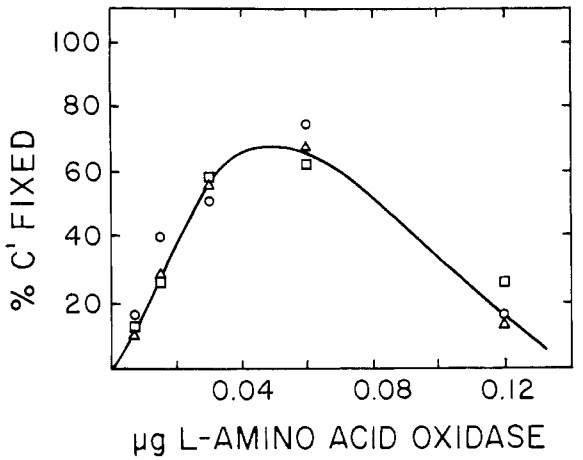

FIGURE 6. Complement fixation curves obtained with active (△), inactive (○), and reactivated (□) L-amino acid oxidase.

(Figure 6). A complement fixation curve obtained anaerobically in the presence of L-leucine also gave results indistinguishable from controls in which this substrate was omitted.

In the course of these studies it was discovered that, on prolonged storage, some dilute enzyme solutions lost their ability to react with the antibody. Figure 7 shows the complement fixation curve obtained with such a solution. The fact that each point on this curve is shifted to the right by about a factor of 4, while the maximal height is the same as that obtained with a fresh solution of enzyme, indicates that approximately three-fourths of the enzyme molecules no longer reacted with the antibody, while the remaining one-fourth reacted with the antibody in the same way as did the original enzyme. When the two enzyme solutions used in the experiment described in Figure 7 were tested for activity, both were found to be equally active. It may therefore be concluded that the loss in immunologic reactivity was due to an alteration in part of the protein molecule (the antibody combining site) rather than complete denaturation.

To prove that the antibody was directed at the enzyme and not at an impurity, the following experiment was carried out. L-Amino acid oxidase solution (0.2 mg/ml) (1 ml) was added to 1 ml of immune rabbit serum. The heavy precipitate which formed immediately was centrifuged, washed, and resuspended in buffer. In a control experiment in which the enzyme was added to the serum of the same rabbit obtained before immunization, no precipitate appeared. The supernatant solution, the precipitate, and the control enzyme were diluted to the same volume, and various aliquots were tested for activity. The results are shown in Figure 8. It may be seen that whereas no activity remained in the supernatant solution, the precipitate exhibited *ca.* 80% of the activity of the control enzyme. Thus, all the enzymatic activity was precipitated by the antibody, and the enzyme–antibody complex was at least 80% as active as the free enzyme.

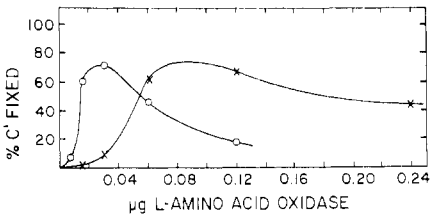

FIGURE 7: Complement fixation curves obtained with a freshly diluted solution of L-amino acid oxidase (o) and with a diluted solution of the enzyme which had been stored for 4 days at 4° (x). The enzyme was diluted with the buffer used in the complement fixation test (see Methods), to a concentration of 7.2 μg/ml.

Since L-amino acid oxidase has been shown to be a glycoprotein (Wellner, 1965), the possibility was considered that the antigenic determinant of the enzyme might be the carbohydrate moiety. A number of monosaccharides, some of which had been tentatively identified in acid hydrolysates of the enzyme, were, therefore, tested as inhibitors of the complement fixation reaction. The following compounds were found to have no inhibitory action under the conditions used, at a concentration of about 2×10^{-3} M: D-glucose, D-mannose, D-galactose, D-ribose, D-glucosamine, D-mannosamine, D-galactosamine, *N*-acetyl-D-glucosamine, and *N*-acetylneuraminic acid. FAD and FMN had no effect at 2×10^{-6} M. By comparison, the concentration of enzyme present in these experiments was 4×10^{-11} M.

Although most of the experiments were carried out with a mixture of the three isozymes (A, B, and C) which occur in rattlesnake venom (Wellner and Meister, 1960a), it was found that the isolated A and C enzymes reacted individually with the antibody to about the same extent as the mixture. In addition, no complement fixation was observed with A in the presence of an excess of C, or with C in the presence of an excess of A. This indicates that the same antibody molecules react with A and C.

Discussion

The experiments described here represent an attempt to obtain some understanding of the structural changes responsible for the reversible inactivation of L-amino acid oxidase. The data obtained by Singer and Kearney (1951) as well as the present results clearly rule out some of the processes known to inactivate enzymes. Thus, there is no loss of FAD or other cofactor, since none must be added to obtain reactivation. There is no dissociation, aggregation, or denaturation of the protein, since no changes in sedimentation coefficient, solubility, or electrophoretic mobility have been observed. The possibility that the inactivation might be associated with a local change in conformation at the active site of the enzyme was therefore investigated, and the results obtained appear to be consistent with this hypothesis.

Free FAD, pH 7.0, has absorption maxima at 375 and

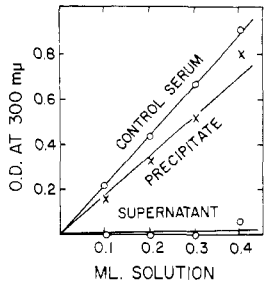

FIGURE 8: Enzymatic activity of the L-amino acid oxidase–antibody precipitate. The activity, represented on the ordinate, was measured by the borate–enol-phenylpyruvate procedure described under Methods.

450 mμ (Whitby, 1953). When FAD is combined with L-amino acid oxidase, however, corresponding maxima are found at 390 and 462 mμ. This shift in the spectrum, analogous to that found in a number of flavoproteins, is the result of the interaction between the enzyme and its prosthetic group. It may be expected, therefore, that alterations in the mode of binding of FAD by the protein might be accompanied by a spectral change. Such a change in the spectrum of L-amino acid oxidase was observed when the enzyme was inactivated. Although the FAD was still firmly bound to the enzyme and was not removed by extensive dialysis, the spectrum of the inactive enzyme became more like that of free FAD.

The optical rotatory dispersion data are in agreement with the conclusion that inactivation of the enzyme is accompanied by a change in the environment of FAD. Although the shape of the optical rotatory dispersion curve of L-amino acid oxidase is complex, it may be interpreted qualitatively in terms of what is known about the optical rotation of proteins.

In regions remote from the absorption maxima of an asymmetric chromophore, optical rotatory dispersion curves are plain, and may usually be described by a one-term Drude equation. Thus, proteins which have no chromophores absorbing in the visible region of the spectrum have plain visible dispersion curves. However, when a protein molecule interacts with a chromophore which absorbs light, an anomalous dispersion, or a Cotton effect, may appear. These have been termed extrinsic Cotton effects (for a recent review of this field, see Ulmer and Vallee, 1965). Since the isoalloxazine group of FAD is the only chromophore in L-amino acid oxidase which absorbs light above 320 mμ, it may be concluded that the Cotton effects observed here in the visible and near ultraviolet are associated with the presence of this group.[3] Thus, one may interpret the solid curve in Figure 2 as exhibiting one positive Cotton effect extending from about 570 to 430 mμ, with a point of inflection at 465 mμ; a second positive Cotton effect extending from 420 to 350 mμ, with a point of inflection at about 385 mμ; and a third positive Cotton effect extending from 305 to 265 mμ, with a point of inflection at 285 mμ (shown in Figure 3). Since 320 mμ is in a region of minimal absorption, the part of the curve extending from 305 to 340 mμ is probably a part of the plain dispersion curve of the protein. Although more work is required before an unequivocal interpretation is possible, it appears probable that the Cotton effects with points of inflection at 465 mμ and 385 mμ are associated with the isoalloxazine chromophore.

It should be noted that although these Cotton effects are still present in the inactive enzyme, they are decreased in amplitude. This may reflect the fact that the isoalloxazine groups of FAD are bound less rigidly or less asymmetrically than in the active enzyme. It is also possible that the binding of only one of the two FAD's is altered on inactivation.

The optical rotatory dispersion of FAD itself (Figure 4) is of interest, particularly because of its Cotton effect centered at 375 mμ. The fact that it is absent in FMN suggests that this Cotton effect is associated with interaction between the isoalloxazine chromophore and the ribose of the adenosine moiety of FAD, or interaction between the two heterocyclic ring systems, as suggested by Warshaw *et al.* (1965) for adenylyl-(3′–5′)-adenosine. Perhaps the same interaction also enhances the 275 mμ Cotton effect of FAD, which has a greater amplitude than the corresponding one in FMN. It is also of interest that the 375 mμ Cotton effect of FAD is shifted toward a longer wavelength (385 mμ) in the enzyme, as is the absorption maximum, and is also reversed.

The small changes observed in the rotation of the enzyme at 231 mμ on inactivation suggest that no extensive unfolding or other major conformational change took place. This conclusion is also supported by the results of the immunochemical study. The microcomplement fixation reaction is usually very sensitive to small conformational alterations in an antigen; *e.g.*, it has been possible to detect by this method an immunochemical difference between horse oxyhemoglobin and deoxyhemoglobin (Reichlin *et al.*, 1964). One may, therefore, expect that if inactivation involved a conformational change in the protein as a whole, this would be reflected in a difference in the complement fixation curves. However, no difference was observed.

The fact that the antigen–antibody precipitate was enzymatically active (Figure 8) indicates that the antibody binding site and the active center of the enzyme are located on different parts of the molecule. This conclusion is supported by the finding that it is possible to alter the active site without affecting the antibody binding site or to alter the antibody binding site without affecting the active site.

The results reported here are consistent with the hypothesis that the two flavins of L-amino acid oxidase are part of one active site. If the enzymatic reaction involved the transfer of a hydrogen atom from the substrate to each of the two flavins, it would be ex-

[3] It should be noted that extrinsic Cotton effects are not restricted to visible chromophores, but may also occur in the near ultraviolet or even in the far ultraviolet. In the latter case, however, they may be difficult to distinguish experimentally from the intrinsic Cotton effects associated with asymmetric peptide bond chromophores.

pected that a relative movement of the flavins, which might be associated with a conformational change at the active center, would abolish the ability of the enzyme to react with its substrate. Evidence that a conformational change which affects the environment of the FAD takes place during inactivation was presented above. Although several lines of evidence point to the involvement of both flavins in the catalytic site of L-amino acid oxidase, direct evidence is needed to resolve this question unequivocally.

Acknowledgments

I wish to thank Dr. Maurice Liss for his help in obtaining the antibody and Dr. David Stollar for his invaluable encouragement and advice in the immunochemical studies. I also wish to thank Mr. Melvin B. Hayes for his help in some of the experiments.

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