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RESONANCE RAMAN STUDY ON THE FLAVIN IN THE PURPLE INTERMEDIATES OF D-AMINO ACID OXIDASE

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Resonance Raman (RR) spectra were measured for the purple intermediates of D-amino acid oxidase reconstituted with isotopically labelled FAD's, *i.e.*, [4a-^{13}C]-, [4,10a-$^{13}C_2$]-, [2-^{13}C]-, [5-^{15}N]-, and [1,3-$^{15}N_2$]flavin adenine dinucleotides, and compared with those with the native enzyme. The RR lines around 1605 cm^{-1} with D-alanine or D-proline as a substrate and at 1548 cm^{-1} with D-alanine undergo isotopic shifts upon [4a-^{13}C]- and [4,10a-$^{13}C_2$]-labelling. These lines are assigned to the vibrational modes associated with C(10a)=C(4a)-C(4)=O moiety of reduced flavin, providing the first assignment of RR lines of reduced flavin and conclusive evidence that reduced flavin is involved in this intermediate.

D-Amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] (DAO) is known to catalyze the oxidative deamination of D-amino acids by way of the so-called "purple intermediate," which is characterized by a broad charge-transfer absorption band around 600 nm extending beyond 700 nm (1-6). The catalytic significance or the precise nature of this intermediate has not been fully understood even though it is generally accepted that the intermediate is a charge transfer complex between the substrate

Abbreviations: RR, resonance Raman; DAO, D-amino acid oxidase.

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intermediate, imino acid, and reduced flavin (5,6). We have recently provided, by RR spectroscopy, direct evidence that the intermediate involves an imino acid and that the C=N double bond of the imino acid contributes to the charge transfer complex (7).

Analysis of isotopic frequency shifts of Raman lines is a powerful technique for the assignment of Raman lines, as has been proved in the assignment of RR lines of oxidized flavin (8). For this purpose we synthesized FAD in which various positions of the flavin moiety are labelled with ^{13}C or ^{15}N. They are subsequently reconstituted with apo DAO and RR spectra of the purple intermediate with the reconstituted DAO were measured with excitation in the charge transfer band. We report herein assignment of RR lines derived from reduced flavin, providing conclusive proof that reduced flavin is the constituent of the purple intermediate.

MATERIALS AND METHODS

Porcine kidney D-amino acid oxidase (DAO) and the apoenzyme were prepared according to Shiga *et al.* (9,10) and Tojo *et al.* (11), respectively.

Synthesis of isotopically enriched FAD: Synthesis of ^{13}C- and ^{15}N-enriched riboflavins was essentially according to Yagi *et al* (12,13). [4a-^{13}C]-, [4,10a-$^{13}C_2$]-, [2-^{13}C]-, [5-^{15}N]-, and [1,3-$^{15}N_2$]riboflavins were synthesized with diethyl [2-^{13}C]malonate (90 atom %, Merck Sharp and Dohme, Canada), diehtyl [1,3-$^{13}C_2$]malonate (90 atom %, Merck Sharp and Dohme, Canada), [^{13}C]urea (91.4 atom %, B.O.C. Ltd., UK), sodium [^{15}N]nitrite (99.2 atom %, Merck Sharp and Dohme, Canada), and [$^{15}N_2$]urea (99.9 atom %, B.O.C. Ltd., UK), respectively. Each isotopically enriched riboflavin was converted to FAD with FAD synthetase complex from *Brevibacterium ammoniagenes* by the methods of Nakamura and Tanaka (14) and Spencer *et al.* (15) with modifications.

Apo DAO was reconstituted with isotopically enriched FAD by incubation of FAD samples in slight molar excess with the apoenzyme at room temperature for 30 min (16). Excess FAD was removed by gel filtration through Sephadex G-25, and the protein fraction was concentrated with the aid of a Centriflo CF25 (Amicon). The concentration of the reconsituted or the native enzyme was based on the molar extinction coefficient of 11,300 M^{-1} cm^{-1} at 455 nm.

The purple intermediate of DAO was prepared with D-alanine or D-proline following the previous procedure (7).

RR spectra were measured on a JASCO Raman spectrometer (Japan Spectroscopic Co.) calibrated with indene (17). The excitation was at 632.8 nm with a He-Ne laser (50 mW, Kinmon Electrics, model KLG-103). All the RR measurements were carried out in 0.1 M sodium pyrophosphate buffer, pH 8.3 at room temperature (about 25°C) in the presence of about 2 % (w/v) of ammonium sulfate as an internal standard whose Raman line appears at 981 cm^{-1}. Visible absorption spectra were measured on a Hitachi 557 spectrometer.

RESULTS AND DISCUSSION

Figures 1 and 2 show the RR spectra of the purple intermediates prepared with D-alanine and D-proline, respectively, as substrates and DAO reconstituted with FAD, where designated positions of flavin are enriched. The absorption spectra of DAO in the oxidized state and in the purple intermediates with D-proline and D-alanine as substrates are prensented in the inset of Fig. 1. The RR spectra of the intermediate with native enzyme are also given for comparison. Each reconstituted enzyme exhibited an identical absorption spectrum to that of the native enzyme.

The RR band in the 1680-1710 cm^{-1} region with D-alanine (Fig. 1) and the 1658 cm^{-1} line with D-proline (Fig. 2) have been assigned to the C=N stretching of the substrate intermediates, α-iminopropionic acid and Δ^1-pyrrolidine-2-carboxylic acid, respectively (7). Consistently with these assignments, these signals did not shift upon isotopic labelling in the flavin moiety. On the contrary, the RR line at about 1605 cm^{-1} undergoes distinct isotopic frequency shift of 4 cm^{-1} upon [4a-^{13}C]-labelling (Figs. 1b and 2b). The shift is even greater (15 cm^{-1} and 20 cm^{-1} with D-alanine and D-proline, respectively) upon [4,10a-^{13}C]-labelling (Figs. 1c and 2c). Similar shifts were observed in the 1548 cm^{-1} line with D-alanine (Fig. 2). Namely, this line was shifted moderately (2 cm^{-1}) upon [4a-^{13}C]-labelling (Fig. 1b) and was shifted further down (7 cm^{-1}) upon [4,10a-^{13}C]-labelling (Fig. 1c). The corresponding line was decreased in intensity when D-proline was used as the substrate (Fig. 2) such that the isotopic frequency shift could not be definitely estimated. No appreciable change was observed in any of the RR lines with [2-^{13}C]-, [5-^{15}N]-, or [1,3-$^{15}N_2$]-labelling (Figs. 1d-f and 2d-f).

The results described above clearly indicate that the RR lines around 1605 cm^{-1} and 1548 cm^{-1} are both derived from the flavin moiety and lead to the assignment of these lines to the vibrational mode

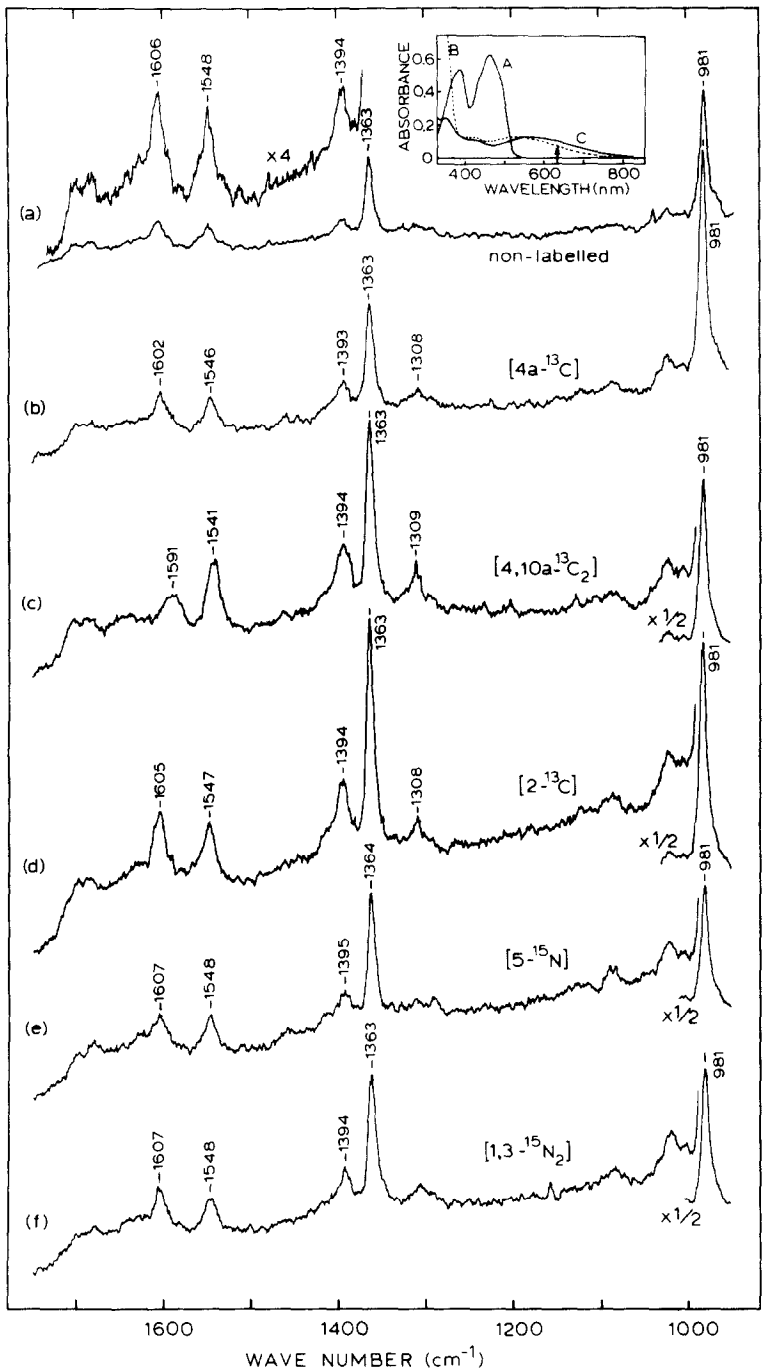

Figure 1. RR spectra of the purple intermediate with D-alanine and native DAO (a) or DAO reconstituted with FAD's isotopically enriched in the flavin moiety (b-f). Positions enriched in the flavin moiety are indicated in the figure. Concentrations were: DAO, 0.75 mM (a), 0.43 mM (b), 0.52 mM (c), 0.54 mM (d), 0.64 mM (e), 0.44 mM (f), D-alanine, 110 mM (a-f); pyruvate, 54 mM (a), 74 mM (b,e,f), 148 mM (c,d); ammonium sulfate, 0.16 M (a), 0.17 M (b-f). The inset shows the visible absorotion spectra of DAO in the oxidized state (A) and in the purple intermediates with D-alanine (B) and D-proline (C) as the substrates in 0.1 M Na-pyrophosphate buffer, pH 8.3. Concentrations were: DAO, 55 μM (A-C); ammonium sulfate, 0.16 M (A-C); D-alanine, 91 mM (B); pyruvate, 54.5 mM (B); D-proline, 91 mM (C). The arrow indicates the excitation wavelength for the RR spectra.

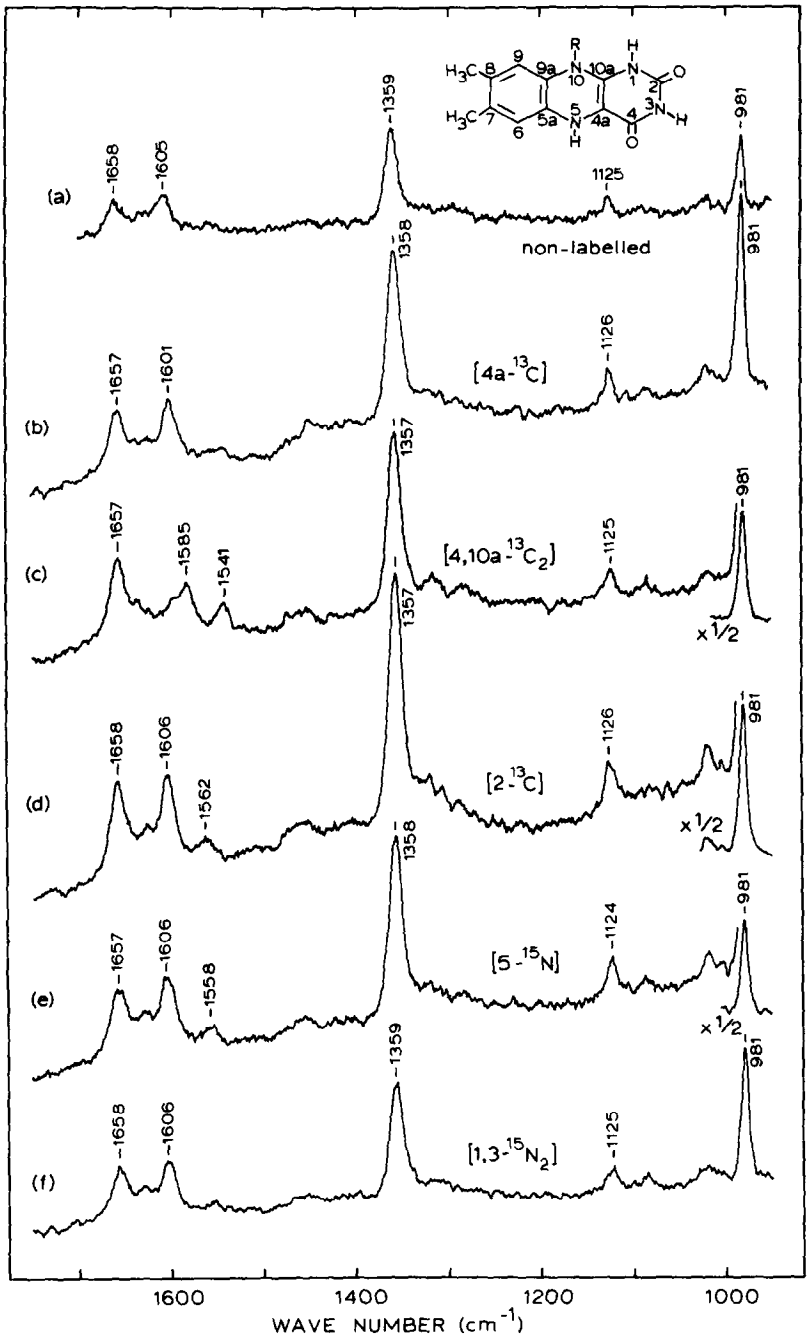

Figure 2. RR spectra of the purple intermediate with D-proline and native DAO (a) or DAO reconstituted with FAD's isotopically enriched in the flavin moiety. Positions enriched are indicated in the figure. Concentrations were: DAO, 0.52 mM (a,e), 0.48 mM (b,f), 0.58 mM (c,d); D-proline, 86 mM (a), 111 mM (b-f); ammonium sulfate, 0.12 M (a), 0.17 M (b,e,f), 0.18 M (c,d). The inset shows the numberings of atoms of reduced flavin.

involving the C(10a)=C(4a)-C(4)=O conjugated system of reduced flavin in agreement with our previous prediction (7). The resonance enhancement

of the lines originated from this portion of reduced flavin when excited in the charge transfer band implies that reduced flavin particiaptes in the charge transfer interaction at this site of reduced flavin. That the reduced flavin is involved in this particular intermediate is also supported by the distinct pattern of the RR spectra of this intermediate from those of oxidized or semiquinoid flavin, as pointed out previously (7). In addition, the possibility that the 1605 cm^{-1} line observed here might correspond to the 1617 cm^{-1} line of the semiquinoid form of flavin (18) is ruled out by the finding that the 1617 cm^{-1} line of the semiquinoid form was not shifted upon [4a-^{13}C]- or [4,10a-$^{13}C_2$]-labelling (Nishina, Y. *et al.*, unpublished results). The 1605 or 1548 cm^{-1} line does not correspond to the 1580 cm^{-1} line of the oxidized flavin, since the latter is susceptible to isotopic shift upon [5-^{15}N]-labelling (8) in contrast to the former (Figs 1 and 2). It is emphasized that the present study provides the first assignment of RR lines derived from reduced flavin and that the RR spectra of reduced flavin were obtained for the first time in our previous (7) and present studies. It is interesting to note that the charge transfer interaction involving oxidized flavin is localized in the N(5)=C(4a)-C(10a)=N(1)-C(2) region (19-23) in close similarity to the region of reduced flavin for the charge transfer interaction revealed in this report. The strong RR line around 1360 cm^{-1} observed with either D-alanine (Fig. 1) or D-proline (Fig. 2) was not shifted to any detectable extent by labelling the flavin moiety at any of the positions examined. We are currently investigating the origin of this line with both enzyme and model systems.

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