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On the Topochemistry of the Active Site of Bovine Liver Monoamine Oxidase B

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ABSTRACT. Fluorescence and electron spin (spin labelling) resonance spectroscopy were used to verify the nature of the active site of bovine liver monoamine oxidase B. Fluorescence polarization studies demonstrated that the flavin moiety moves with a certain degree of mobility around the covalent bond.

The ESR spectrum of the enzyme labelled with an N-ethylmaleimide spin label shows components corresponding to weakly and to strongly immobilized labels. The binding of the probe to the active site over a temperature range of 5-50°C was studied. From the change of the ESR spectra, it is suggested that the active enzyme is in thermal equilibrium between two conformational states, and this conformational change involves only a short region of the active site of the enzyme. It is also suggested that the two conformational states may involve not only the active site sulfhydryl group, but also the environment surrounding the flavine moiety of the enzyme. A model of the two conformational states of the active site of the enzyme is proposed.

Monoamine oxidase (MAO) is postulated to play an important role in controlling the levels of certain neurohormones and other pharmacologically important amines (Udin 1974, Schacht and Leven 1977). This involvement has stimulated considerable interest in the physicochemical and enzymatic properties of the enzyme.

The enzyme has a molecular weight of 104,000 per protomer and is made up of two subunits each of which has a molecular weight of 52,000 (Chaung *et al.* 1974) and

there are no disulfide linkages between the subunits. There appears to be one mole of FAD per two subunits, which is covalently attached to the protein (Orleand 1971). From the effect of pH on the kinetic properties of the enzyme (Igaue *et al.* 1967), it was concluded that a cysteine residue in the enzyme is catalytically important in the cleavage of the C–N bond in the substrate. Gomes *et al.* (1976) concluded that there are possibly two essential cysteine residues in the active site per protomer from the reaction of the enzyme with mercurials and [1]DTNB. Yasunobu *et al.* (1979) have studied the reactivity of various alkylating agents CH_3HgI, DTNB, NEM, iodoacetic acid with both the essential and non-essential cysteine residues. They concluded that the order of reactivity of the reagents were CH_3HgI > DTNB > NEM > iodoacetic acid. They found hydrophobic interactions between the enzyme and alkylating agents to be important. As a first step toward a three dimensional model of bovine liver monoamine oxidase B, Zeidan *et al.* (1980) have embarked on a specific study of the environment of the essential sulfhydryl residue using ESR spin labelling techniques. The details of this aspect of MAO research has been elaborated in detail elsewhere (Zeidan *et al.* 1980). Briefly, the enzyme was first converted to the S-B-carboxymethyl cysteine derivative in which the non-essential cysteine residues were derivatized. Then, the remaining two essential cysteine residues were reacted with spin labelled N-ethyl maleimide. This resulted in the attachment of the nitroxide group to the essential sulfhydryl residues. Our results suggested that the essential sulfhydryl groups are a part of the active site, and that the active site is a shallow crevice, Zeidan *et al.* 1980. As a continuation in our efforts to verify the nature of the active site, we have now embarked upon a specific study to probe the nature of the active of the enzyme conformational changes induced by temperature.

In this communication, we report the successful use of ESR-spin labelling techniques in probing the two different conformational states of the active site of bovine liver monoamine oxidase B.

Experimental

A. Material

Highly purified enzyme was prepared by the method of Yasunobu *et al.* (1979). The specific activity of the enzyme was 9500 units/mg. Iodoacetic acid was recrystallized from chloroform. Nitroxide spin labelled N-ethyl-maleimide supplied by Syva Co. Other common reagents used were of reagent grade quality.

Modification of Bovine Liver Monoamine Oxidase

The enzyme was reacted first with iodoacetic acid as described previously (Zeidan *et al.* 1980) for 6hr, and was then distilled by passing through 2-3 cm of "Sephadex" G-25. The modification of the non-essential sulfhydryl residues was monitored by following the enzymatic activity and amino acid analysis as described by Zeidan *et al.*

1. DTNB: 5,6-dithiobis-(2-nitrobenzoic) acid.

(1980) The enzyme was assayed for activity by spectrophotometric procedure of Tabor *et al.* (1954) in which the formation of benzaldehyde is determined at 250 nm.

Modification of [2](Cys-Cm)$_6$• Enzyme Derivative Using Nitroxide Spin Labelled N-ethyl Maleimide

Modification of [2](Cys-Cm)$_6$• enzyme with nitroxide spin label N-ethyl maleimide was carried out as described previously (Zeidan *et al.* 1980). The reaction was then allowed to proceed with gentle stirring at room temperature and under anaerobic conditions for 15hr. It was exhaustively dialyzed against 0.05 M potassium phosphate pH 7.5.

B. Methods

Electron paramagnetic resonance (ESR) measurements were recorded at various temperatures 0-50°C on a Varian E-4 spectrometer. The field setting was 3415 gaus, the microwave frequency 9.5 GHz and a modulation amplitude of 4.00 gaus was used throughout.

Spin label concentrations were quantitated by double integration of the ESR spectra using a computer interfaced with the ESR spectrometer. The spin label 2,2,6,6-tetramethyl-4-pyridinol oxyl (Tempol) was used as the standard to calibrate the spin label concentrations. One important application of the computer in ESR is special subtraction which makes possible the resolution of a complete ESR spectrum into component parts: strongly bound and partially immobilized spin labels.

Spectral subtraction requires that a spectrum of one or more of the components be obtained separately. Reference or standard spectra were obtained by taking the spectra of a standard maleimide spin label of known concentration and dissolved in 95% glycerol at different temperatures. This method gives rise to ESR spectra of different immobilizations. (Results are not shown).

The digitalized spectra of the reference and the composite spectra were placed in different memory locations in the computer. If necessary, the computer shifts the spectra relative to each other so that the desired peaks are in register. Successive increments of the reference spectrum are subtracted until the component being subtracted from the composite disappears.

We recorded fluorescence spectra using a Perkin-Elmer MPF 2A spectrofluorometer ambient temperature and corrected fluorescence spectra with a Perkin-Elmer MPF 3 spectrofluorometer. Fluorescence polarization measurements were obtained on the MPF 2A fluorometer with the standard polarization accessory. Studies in temperature dependence entailed circulating thermostated water around a cell holder.

The quantum yield was determined using flavin in 0.05 M potassium phosphate pH 7.4 as a standard. Equation 1 gives the ratio of quantum yield, Q, as a function of the

2. (Cys-Cm)$_6$: ß-carboxymethyl cysteine a modified enzyme was prepared by the reaction of the six non essential sulfhydryl residue with 0.05M iodoacetic acid for 6 hours at pH 7.4.

area of the corrected emission spectrum, Fi, and the absorbance at the exciting wavelength, A_1, for two different fluorescent labels.

$$\frac{Q_2}{Q_1} = \frac{F_2A_1}{F_1A_2}$$

For measuring quantum yields, the absorbance at the exciting wavelength, 450 nm, was less than 0.005. The area under the corrected emission spectra were calculated using a computer.

For polarization measurements, a correction was made for unequal transmission of horizontally and vertically polarized light by the emission monochromator grating. We calculated fluorescence polarization P from the relation:

$$P = \frac{I_v - GI_{vh}}{I_v + GI_{vh}}$$

where I is the observed fluorescence intensity and the first and second subscripts refer to the orientation of the polarizer and analyzer, respectively, and G is a correction factor for polarization introduced by the emission monochromator grating and is given by $G = I_{vh}/lHH$. All fluorescence measurements were made with a microcuvette of 1.5 mm effective path length to minimize any corrections applied for inner filter effects. For the temperature course plot for the depolarization of monoamine oxidase B fluorescence (Fig. 9), a solution of 4.0×10^{-6} M monoamine oxidase B in 0.05 M potassium phosphate buffer was excited with polarized light at 3.60. The emitted light was analyzed with the monoromator set at 470 nm. The data were analyzed by computer service to determine the scope and intercept of the line defined by the points according to a mean least squares approximation.

Results and Discussion

Although the enzyme monoamine oxidase (MAO), monoamine O_2: oxidoreductase (deaminating: EC 1.4.3.4), has been the subject of intensive study in many laboratories for some two decades now, there are still many unanswered questions in regards to details of its structure and mechanism of action and considerable controversy over the interpretation of that which is known. Central to this controversy is the question concerning the nature of the active site of the enzyme. Several investigators (Tipton and Coret 1979, Houslay 1978) have presented evidence of two or more independent catalytic sites with different specificities on the same molecular species. The idea that there might be two active sites on a single enzyme molecule has been proposed by White *et al.* (1977), Mantle *et al.* (1975) and egashiro *et al.* 1978.

One of the approaches took to investigate the nature of the active site was the use of an electron spin resonance (ESR) spin labelling technique to probe the active site of the enzyme (Tabor *et al.* 1954). From our previous investigations, we concluded that

the active site is a shallow crevice (Zeidan *et al.* 1980). The ESR spectrum of maleimide[3] spin labelled 110* bound to the active site sulfhydryl group exhibits two types of immobilization, one very strong and the other partially immobilized type represented by the arrows at room temperature (Fig. 1).

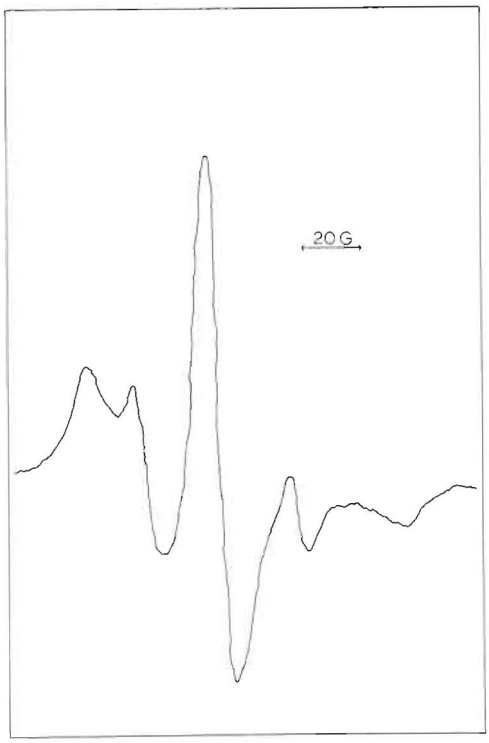

Fig.1. ESR spectrum of the $(Cys\text{-}Cm)_6$ — $(Cys\text{-}ISL)_2$ derivative with spin label N-ethylmaleimide 110. The $(Cys\text{-}Cm)_6$-enzyme derivative was prepared as described in the experimental procedure section. About 1.0 mg of this derivative was inerbrated with 3 maleimido-2,2,5,5, tetramethyl-1-pyrolidinyloxyl spin label (spin label 110) in 1.0 ml of 0.05 M potassium phosphate, pH 7.5, and reacted for 18 hr at 25°C in nitrogen atmosphere. The enzyme solution was exhaustively dialyzed and the ESR spectrum was taken at room temperature as described in the experimental section.

The total bound spin label was calculated and found to be only 1-1.2 mole/mole of monomer of carboxylated protein, suggesting that only the active site sulfhydryl group was labelled. The two degrees of immobilization suggest two different environments. The strongly immobilized peak was calculated by spectral subtraction method and found to be 80% and the partially immobilized peak was 20% at room temperature

3. Spin label 110:3-maleimide-2,2,5,5, tetramethyl-1-pyrolidonyloxyl.

(Fig. 2). Since the stoichiometry of the total spin label never exceeded 1-1.2 mole/mole of monomer of carboxymethylated protein and since amino acid analysis of the carboxymethylated enzyme indicates that carboxymethylation of only the cysteine residue occurs and none of the histidine nor lysine residues are carboxymethylated under these conditions, it is reasonable to suggest that this heterogeneity with spin label maleimide in fact reflects an intrinsic property of the native enzyme.

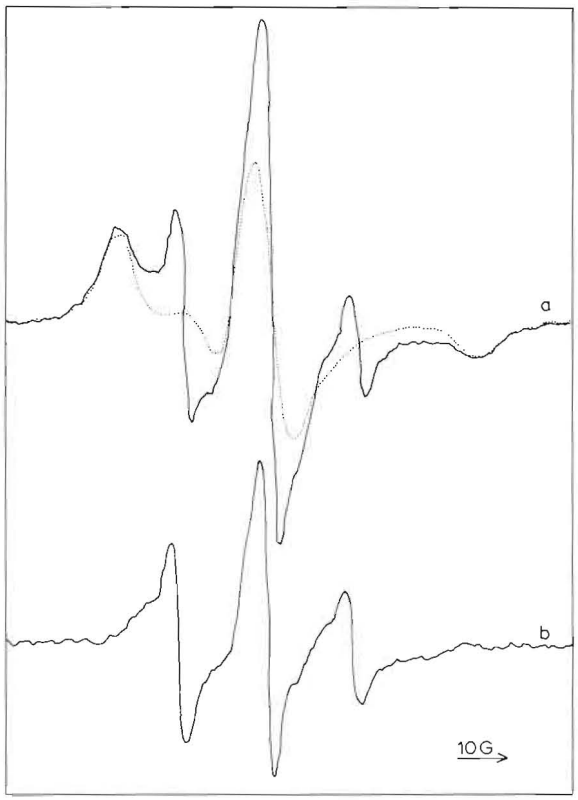

Fig.2. Resolution of the ESR spectrum of ISL 110 bound to $(Cys\text{-}Cm)_6$ bovine liver monoamine oxidase B. The spectral subtraction was done on the Varian E-4 on line computer a) subtraction of the strongly immobilized component (dotted line) from the composite. b) The difference spectrum which represents a partially immobilized component.

In order to investigate the phenomenon of the heterogeneity of the active site sylfhydryl residues with maleimide spin-labels, the binding of the probe to the active site of monoamine oxidase was studied over a temperature range of 5-50°C and spectra and the results of this investigation are presented in Fig. 3, 4. At 5°C the spectrum is

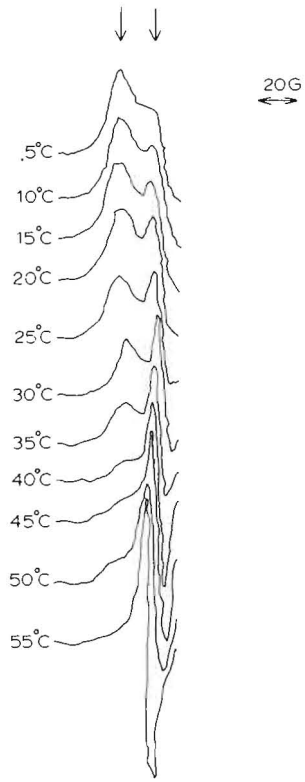

Fig.3. The effect of temperature on the ESR spectrum of bovine liver monoamine oxidase B a) 5°C, b) 10°C, c) 15°C d)20°C, g) 40°C, h) 50°C.

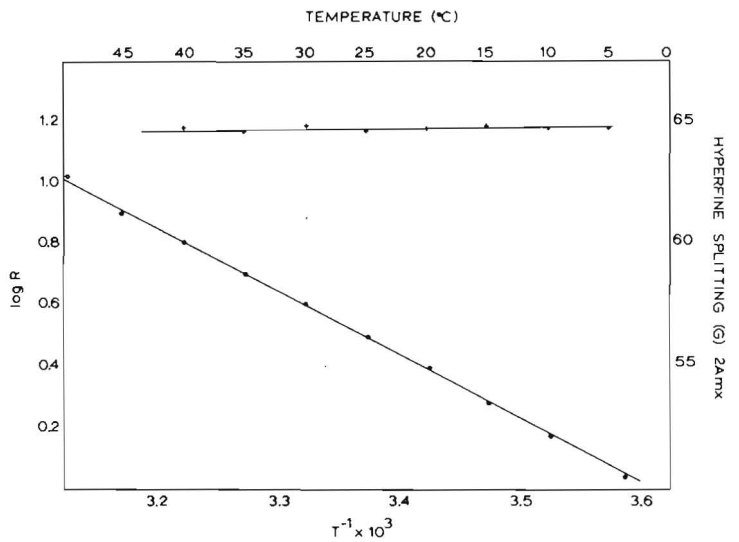

Fig.4. a) The ratio of the mobilized and immobilized, spin population is plotted against temperature. Data were obtained from Fig.1, b) The extent of immobilization (The hyperfire spliting constant 2 Am. is plotted against temperature).

essentially that of strongly immobilized species, Fig. la. By increasing the temperature in the range of 5-50°C, the ratio of strongly to partially immobilized species (judged by the peak height of both populations) has been altered with no change in the extent of immobilization (splitting constant between the low and high field peaks, Fig. 3a-e, Fig. 4, suggesting that the conformational change is very specific to local change, since the correlation time has not been changed. The effect of temperature on the enzymatic activity was studied and the results of this investigation are presented in Fig. 5. It can be concluded from this study that the native enzyme maintained over more than 85% of its activity up to 50°C, then the activity dropped to 25% at 65°C and

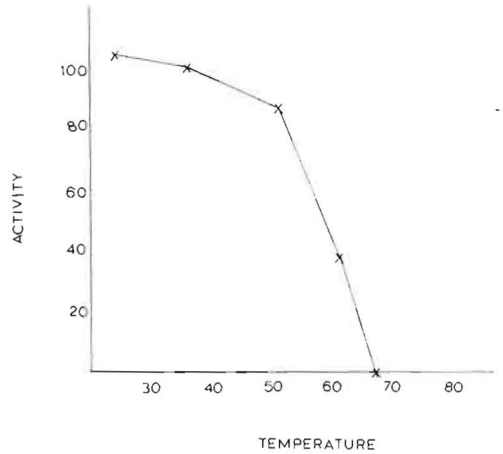

Fig.5. The effect of temperature on the enzyme activity of bovine liver monoamine oxidase B.

finally the enzyme was denatured at 70°C. These results suggest that this local conformational change in the region between 0-45°C involves the native enzyme in its fully active form. The linear semi log plot of ln R versus 1/T over the range 15-45°C which is below the main unfolding transition of Tm, suggest that the native molecule is in thermal equilibrium between two conformational states N and X with Keq K (Fig. 6).

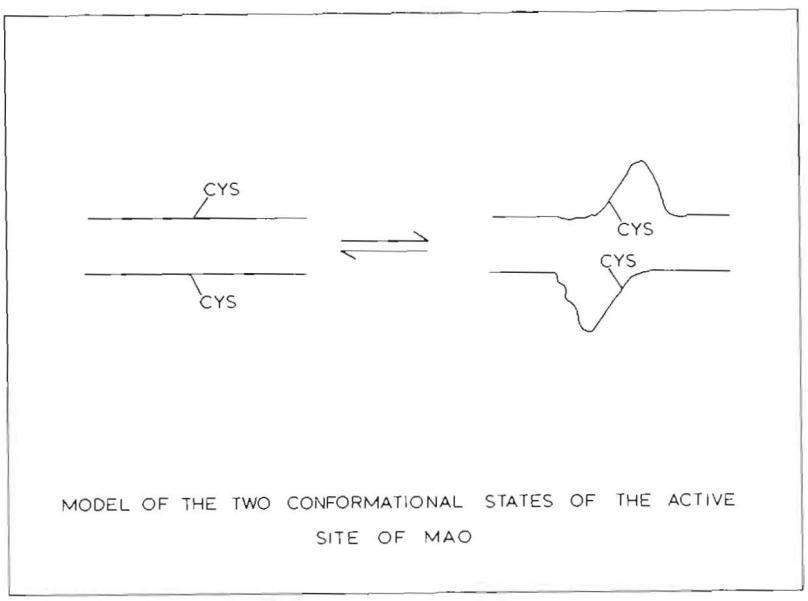

Fig.6. A model for the two conformational states of the MAO enzyme.

In the N-state, the spin label is very rigid on the protein and X is tumbling rapidly. From this plot, one can calculate enthalpy $\triangle H$, and entropy values $\triangle S$ and $\triangle G$ were calculated and found to be 21 cal/mole, and 72 cal/deg-mole, respectively. Only at 52°C the rapid increase in the R was complete. Tm in the X state can be quite flexible in this region of the molecule. The fact that the change in the ratio of the strongly to partially immobilized species was reversible adds a supportive evidence of the nature of the equilibrium between the two states N, X.

Thus, the change observed in going from 5-50°C could be interpreted as the reactive enzyme is in thermal equilibrium between two states N, X (Fig. 6) and increasing the temperature in this range induces a conformational change in the protein in the moiety of the sulfhydryl residue, resulting in changing the ratio of partially immobilized spin to strongly immobilized spin. This conformational change need not involve the whole active protein but may merely be a local conformational change at the sulfhydryl active site, and its surrounding environment.

The amino acid composition of the enzyme was determined previously by Minamuria and Yasunobu (1978) and was found to be 481 amino acid residue per 52,000 g of protein per subunit of the enzyme. $\triangle H$, $\triangle S$ between N, X states are very small suggesting a complete unfolding of only a short region of about 25-30 residues out of 480 (Lehrer and Gracefa 1980).

Temperature Dependence of Enzyme Activity, and of Relative Fluorescence (Quantum Yield (Q rel) of the Flavin).

The effect of the temperature on the catalytic activity of the enzyme was studied and the results of this investigation are shown in Fig. 7. The temperature transition of en-

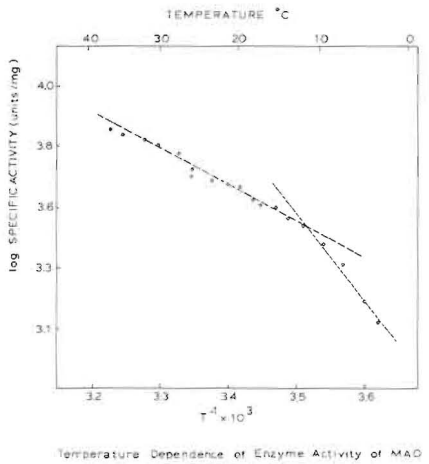

Fig.7. Temperature dependence of enzyme MAO activity.

zyme activity can be seen from the profile of Arrhenius plot in the range of 15-20°C. This temperature transition change suggests a change in catalytic activity of the enzyme in this region.

In another experiment, we investigated the temperature dependence of relative fluorescence quantum yield (Q rel) of the flavin moiety, and the results of this experiment is shown in Fig. 8. The quantum yield of FAD in the enzyme against riboflavin (in 0.05 M potassium phosphate) was about 1.6% at 5°C and decreased with increasing the temperature (from 5 to 30°C). From the profile of Arrhenius plot, the temperature transition was 15 to 18°C. At this transition temperature range (15-18°C), there was a conformational change occurred in the local environment surrounding flavin moiety of the enzyme, and this local conformational change reflects the change of the catalytic activity of the enzyme.

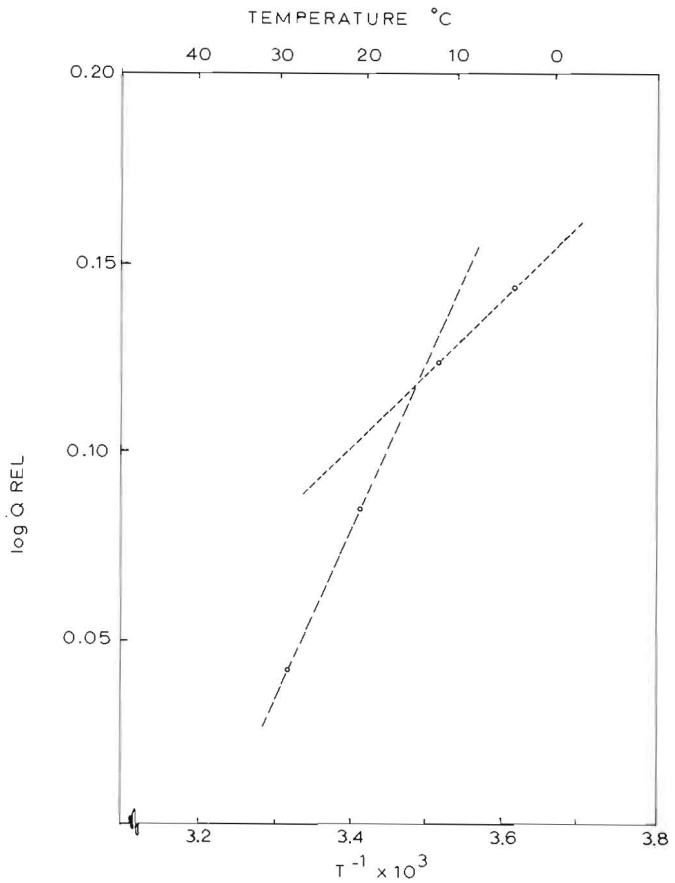

Fig.8. Temperature dependence of relative fluorescence quantum yield (Q rel) of the FAD.

Fluorescence Polarization

The degrees of fluorescence polarization is a function of the geometrical orientation of the exciting light. Absorbing dipole, emitting dipole, and changes of dipole orientation while in the excited state.

Many factors can increase the extent of depolarization; the two most important factors are the motion of the absorber and energy transfer between like chromophores. If the emitter is rotating very rapidly, *i.e.*, if there is a substantial change in orientation during the life time of the excited state, the polarization will be further reduced.

This is an important phenomenon because the extent of this type of depolarization is affected by temperature, solvent viscosity and shape of the molecule containing the emitter. The effect of the temperature on the fluorescence polarization of the flavin was studied and the results of this investigation is shown in Fig. 9.

The results suggested a temperature transition around 15°C, which support a conformational change in the local environment of the flavin moiety of the enzyme at this temperature range. It was of interest to us to determine the geometric orientation and

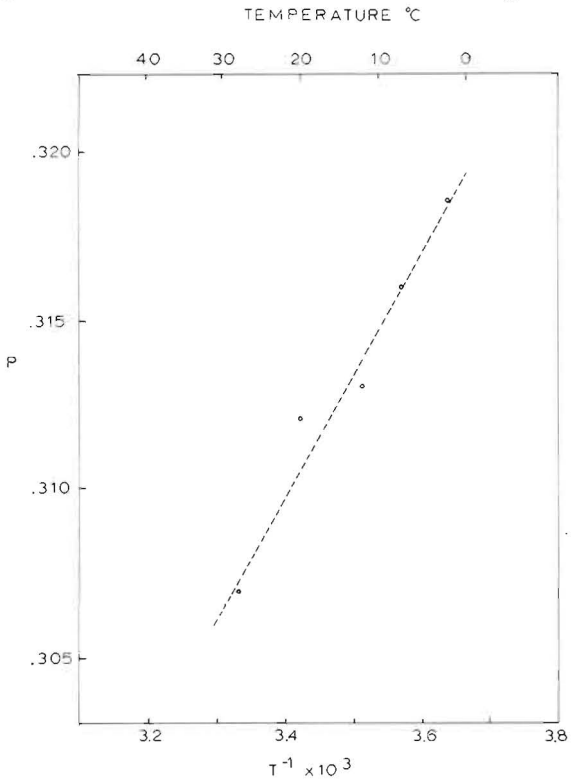

Fig.9. Temperature course plot for the depolarization of monoamine oxidase B fluorescence.

the motion of the FAD moiety. The value of polarization degree was found to be 0.318 at 3.0°C. These results suggest to us that the flavin moiety of the enzyme can move with a certain degree of mobility around the covalent bond and is not completely immobile and tightly fixed to the enzyme such as the flavin moiety of succinic dehydrogenase enzyme.

From our recent studies on the active site of monoamine oxidase by fluorescence technique, we concluded that increasing the temperature in the range from 5-45°C induces a local conformational change in the local environment surrounding the flavine moiety of the enzyme at the transition temperature 15-40°C (Fig. 8).

The ESR spin labelling investigation reported here with the activity studies seems to support the suggestive evidence of the thermal equilibrium of the active site of the enzyme within 5-40°C. The results of our present investigation suggest that this small local conformational change at the active site may not only involve the sulfhydryl site, but also the flavine moiety of the enzyme. Our research now is in progress to determine the role of the flavine moiety and the sulfhydryl residue in the mode of action of the enzyme.

Model of Two Conformational States of the Active Site of Bovine Liver Monoamine Oxidase B.

Our previous and present investigations led us to believe that the active site of MAO is a hydrophobic shallow crevice. Furthermore, we believe that there are different locations of the binding area of the substrate within the cavity, as in so many cases MAO substrates essentially consist of two parts, a reactive component and a hydrophobic moiety (which may or may not carry polar residue). The latter not only contributes a considerable part of $\triangle G$ in the MAO-substrate interaction, but it also modulates the responsiveness of the reactive part. The correct positioning requires the loss of translational and rotational motions of entropy. A major part of the entropy loss is likely to be brought about if any part of the substrate becomes anchored to the active site. Once the anchoring has taken place, free translation of the substrate relative to the enzyme is no longer possible and only internal motions need to be moved slightly to bring the reactive groups into the correct position. This anchoring effect is needed to bring about the highly improbable positioning of reactive groups in the optimum manner. We assume that the proton donor, together with the electron acceptor are differently located with respect to the hydrophobic area. Our model makes it plausible that small changes in the distance between the essential components of the active site could markedly shift the active site between two conformational states X, N (Fig. 6).

In summary, our results conclude the following:

1) The native enzyme is in thermal equilibrium between two confromational states.

2) This conformational change involves only a short region of the active site.

3) The local conformational change may involve both the active site sulfhydryl residue and the flavine moiety of the enzyme.

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References

Chuang, H. Y. K., Patek, D. R. and **Hellerman, M.** (1974) Mitochondrial monoamine oxidase inactivation by pargyline. Adduct formation, *J. biol. Chem.* **249:** 2384-2391.

Egashiro, T., Kuriowa, Y. and **Kamijo, K.** (1978) Multiple catalytic sites of rat brain mitochondrial monoamine oxidase B, *Arch. Biochem. biophys.* **191:** 714.

Gomes, B., Kloepfer, H. G., Oi, S. and **Yasunobu, K. T.** (1976) The Reaction of sulfhydryl Reagents with Bovine hepatic monoamine oxidase, *Biochim. biophys. Acta.* **438:** 347-357.

Houslay, M. D. (1978) Lysolecithin is a selective reversible inhibitor of mitochondrial monoamine oxidase, *Biochem. Pharmacol.* **27:** 1287-1288.

Igaue, I., Gomes, B. and **Yasunobu, K. T.** (1967) Beef mitochondrial monoamine oxidase, a Flavin dinucleotide enzyme, *Biochem. biophys. Res. Commun.* **20:** 563-570.

Lehrer, S. S. and **Gracefa, P.** (1980) The excimer Florescence of Pyrene-labelled tropomycin. A probe of conformational dynamic, *J. biol. Chem.* **255:** 11296-11300.

Mantle, T. J., Wilson, K. and **Long, R. F.** (1975) Kinetic studies of membrane bound rat liver monoamine oxidase, *Biochem. Pharmacol.* **24:** 2039-2046.

Minamuria, N. and **Yasunobu, K. T.** (1978) Bovine Liver Monoamine oxidase. Modified purification procedure and Preliminary evidence for two subunits and one FAD, *Arch. Biochem. Biophys.* **189:** 481-489.

Orleand. L. (1971) Role of acidic phospholipids' in the binding of monoamine oxidase to the mitochondrial structure, *Archs Biochem. Biophys.* **142: 710-714.**

Schacht, V. and **Leven, M.** (1977) *Depressive Disorders Symposium,* Rowe, Publisher F. K. Schattaner Verlag, Stutgart, New York, 21-40.

Tabor, C. W., Tabor, H. and **Rosenthal, S. M.** (1954) Spectrophotometric determination of monamine oxidase, *J. Biol. Chem.* **208:** 645.

Tipton, K. F. and **Coret, L. (1979)** ***in:*** **Singer, T. P., Von Korff, R. W.** and **Murphy, D.L. (eds)** *Monoamine Oxidase: Structure, Function and Altered Functions,* Academic Press, pp. 87-99.

Udin, E. (1974) *Neuropsychopharmocology of Manoamines and Their Regulatory Enzymes,* Raven Press, New York, 1-471.

White, H. L., Glassman, A. T. and **Wu, I.** (1977) Multiple binding sites of human brain and liver monoamine oxidase substrate specificities, selective inhibitors and attempt to separate enzvme forms, *J. Neurochem.* **29:** 987-997.

Yasunobu, L., Watanabe, K. and **Zeidan, H.** (1979) *in* **Singer, T. P., Von Korff, R. W.** and **Murphy, D. L. (eds)** *Monoamine Oxidase: Structure, Function and Altered Functions,* Academic Press, pp. 251-263.

Zeidan, H., Watanabe, K., Piette, L. and **Yasunobu K.** (1980) *in:* **Mayammiya, L. B.** and **Yasunobu, K. (eds)** *Frontiers in Protein Chemistry,* Elsevier. North Holland Press, pp. 131-148.

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حول طبوكيميائية الموقع النشط في إنزيم أحادي أمين أكسيداز ب لكبد البقر

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استخدمت أجهزة الفلورية ومطيافية رنين الإلكترون الحر في التحقق من طبيعة الموقع النشط في إنزيم أحادي أمين الأكسيداز ب لكبد البقر. وقد أثبتت دراسات استقطاب الفلورية أن الشطر الفلافيني يتحرك بحرية كبيرة حول الرباط المشترك.

ويوضح طيف رنين الإلكترون الحر ـ المتعلق بمجموعة كيميائية فعالة ولازمة للتفاعل مع حمض أميني يحتوي على كبريت في صورة مختزلة ـ أن هناك حالتان شكليتان إحداهما تدل على أن الإلكترون الحر محدود الحركة، والثانية تدل على أنه لا يتحرك بل هو ملتصق بمكان عميق من البروتين. وقد تناولت الدراسة علاقة ارتباط الإلكترون الحر بالجزء الفعال من الإنزيم مع تغير درجة الحرارة من ٥ـ٥٠ درجة مئوية.

ومن التغير في أطياف رنين الإلكترون الحر، تشير النتائج إلى أن الجزء النشط من الأنزيم في حالة اتزان حراري بين حالتين شكليتين، وأن هذا التغير الشكلي يقع في منطقة صغيرة من الجزء النشط من الإنزيم. أضف إلى ذلك، أن التغير لا يشمل الحمض الأميني المحتوي على الكبريت في صورة مختزلة فحسب، بل أيضاً الوسط الذي يتحرك في الشطر الفلافيني أيضاً.

ويقترح الباحث أنموذجاً لكلتا الحالتين الشكليتين للجزء النشط من الإنزيم.