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INVESTIGATION OF THE NATURE OF *o*-PHTHALALDEHYDE REACTION WITH OCTOPINE DEHYDROGENASE

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The effect of *o*-phthalaldehyde on octopine dehydrogenase inactivation has been studied. *o*-Phthalaldehyde binds to the proximal cysteine and lysine residues of the enzyme leading to the formation of isoindole derivative. Double inhibition studies with *o*-phthalaldehyde and *p*-chloromercuricphenyl sulfonic acid have indicated that *o*-phthalaldehyde does not bind to the functional cysteine present at the active site. Protection experiments have shown that L-arginine prevented *o*-phthalaldehyde inactivation. This could be only due to the reaction of the amino group of L-arginine with *o*-phthalaldehyde as per the mechanism proposed elsewhere since L-arginine cannot bind to the enzyme prior to NADH. Other substrates such as pyruvate or NADH could not prevent the *o*-phthalaldehyde reaction with the enzyme. Fluorescence spectral studies demonstrated that in the presence of externally added amino acid no isoindole derivative formation occurs. However, a characteristic isoindole derivative is formed in the presence of β-mercaptoethanol although the enzyme does not lose its activity. This indicated that *o*-phthalaldehyde can bind with lysine of the enzyme and thiol of externally added β-mercaptoethanol. Pyridoxal 5′-phosphate, a lysine specific reagent also binds to the enzyme giving the characteristic absorption and fluorescence peak at 325 nm and 395 nm respectively. However, no loss of enzyme activity was observed. On the basis of these experiments we would suggest that *o*-phthalaldehyde binds to non-essential cysteine and lysine residues present in close proximity which results in conformational changes leading to enzyme inactivation.

KEY WORDS: *o*-phthalaldehyde, octopine dehydrogenase, isoindole derivative.

INTRODUCTION

o-Phthalaldehyde, a fluorescent bifunctional compound has been found to be an important reagent in a number of qualitative and quantitative studies.[1–6] It is extensively used as a fluorogenic reagent for determination of the number of amino acids in proteins and enzymes.[5] More recently it has also been used as an affinity reagent for several enzymes such as aldolase,[7] adenosine cyclic 3′ 5′-monophosphate dependent protein kinase,[8] phosphoenolpyruvate carboxykinase,[9] taka-amylase[10] and malate dehydrogenase.[11]

Many studies have been reported on the *o*-phthalaldehyde reaction with primary amines and thiol containing compounds.[12–15] However, little is known on the nature

* Correspondence

of *o*-phthalaldehyde binding with proteins or with functional groups of enzymes in the presence of amino or sulfhydryl group donors.[16]

Octopine dehydrogenase performs a similar function as lactate dehydrogenase in lower animals.[17] In our earlier report it was shown that cysteine is involved in the catalytic reaction of octopine dehydrogenase.[18] However, *o*-phthalaldehyde binds to the cysteine and lysine residues which are not present at the catalytic center of octopine dehydrogenase. We also showed that one of its substrate, L-arginine, protected the enzyme activity against *o*-phthalaldehyde inactivation.[19] This is apparently contradictory with the results of the previous kinetic studies which have indicated that octopine dehydrogenase has an affinity to bind only with NADH initially.[20,21] In order to resolve the discrepancy, a detailed investigation has been undertaken to study the effect of L-arginine and other amino and thiol compounds on the *o*-phthalaldehyde reaction with octopine dehydrogenase. The evidence presented here clearly demonstrated that binding of *o*-phthalaldehyde takes place at a nonsubstrate binding region of this enzyme and conformational deformations is the cause for the inactivation of octopine dehydrogenase.

EXPERIMENTAL PROCEDURE

Materials

Octopine dehydrogenase, NADH and L-arginine were purchased from Sigma Chemical Company USA. Sodium pyruvate was purchased from Boehringer Mannheim GmbH, Germany. Other reagents *i.e.* *o*-phthalaldehyde, *p*-chloromercuricphenyl sulfonic acid, glycine, 5′ *p*-fluorosulfonylbenzoyl adenosine, dithiothreitol, pyridoxal 5′-phosphate, β-mercaptoethanol and chemicals used for buffer preparation were also obtained from Sigma Chemical Company USA. All other chemicals used were of highest purity grade commercially available.

Methods

Enzyme assay The enzyme activity was measured by monitoring the decrease in absorbance at 340 nm With respect to the disappearance of NADH. These measurements were carried out on Gilford spectrophotometer, Model 260 equipped with a Gilford recorder. The assay mixture was prepared as reported previously.[19]

Inactivation studies on octopine dehydrogenase *o*-Phthalaldehyde was first dissolved in 1% distilled methanol before diluting it with the appropriate quantity of assay buffer. For inactivation experiments, octopine dehydrogenase (4 μM) was incubated with the indicated concentration of *o*-phthalaldehyde in 0.1 mM potassium phosphate buffer at pH 7.0. Control experiments were carried out under identical conditions in the absence of *o*-phthalaldehyde.

Pyridoxal 5′-phosphate binding experiments were carried out by incubating the enzyme (0.4 μM) with 53 μM pyridoxal 5′-phosphate for 20 min followed by the reduction with 10 mM sodium borohydride as described elsewhere.[22] Another reagent, 5′ *p*-fluorosulfonylbenzoyl adenosine (8 mM) was dissolved in 15% dimethyl formamide.

This reagent was incubated with 4 μM enzyme in 0.1 mM potassium phosphate buffer (pH 7.0). The residual enzyme activity was measured after 30 min.

Double inhibition experiments The enzyme (4 μM) was first incubated with PCMS for 8 min followed by the addition of *o*-phthalaldehyde and further incubation for 8 min. In another set the order of addition of inhibitors were reversed. Aliquots were withdrawn and added to 20 mM cysteine and 5 mM β-mercaptoethanol solution. The residual activity was then determined. Control sets in the absence of inhibitors or in the presence of only one inhibitor treated with same concentration of cysteine and β-mercaptoethanol were run concurrently.

Spectral measurements

All the fluorescence spectra were recorded at 25°C on a Perkin Elmer Fluorometer (Model LS50B) except for the enzyme pyridoxal 5′-phosphate complex which was recorded on the spectrofluorometer described earlier.[19] Fluorescence emission and excitation spectra were recorded at indicated excitation and emission wavelengths. Absorption spectra were recorded on a Gilford response spectrophotometer (Model Response^{TM}) in cuvettes of 1 cm path length.

RESULTS

Octopine dehydrogenase was incubated with different concentrations of *o*-phthalaldehyde. Enzyme activity was monitored as a function of time of incubation as shown in Figure 1. The rate of inactivation was dependent on the concentration of the reagent used so that a greater loss of enzyme activity was obtained at higher concentrations of *o*-phthalaldehyde. The reaction between *o*-phthalaldehyde and enzyme is irreversible[19] and the treatment of cysteine and β-mercaptoethanol terminates any further reaction of *o*-phthalaldehyde with the enzyme.

The data in Figure 2 show that the inactivation of enzyme in the presence of PCMS was readily reversed by cysteine and β-mercaptoethanol (bar 1,2). However, *o*-phthalaldehyde inactivation was terminated but not reversed in the presence of cysteine and β-mercaptoethanol after 8 min (bar 3,4) and after 16 min (bar 5,6). When *o*-phthalaldehyde was added to PCMS treated enzyme almost complete loss of activity was obtained (bar 7). Further, addition of cysteine and β-mercaptoethanol, reversed only the fraction of the activity that had not been inhibited by *o*-phthalaldehyde (bar 8). similar results were obtained when the sequence of addition was reversed (bar 9). These results indicated that binding of PCMS and *o*-phthalaldehyde takes place at different sites.

In another set of experiment when L-arginine – treated enzyme was first incubated with *o*-phthalaldehyde followed by PCMS, complete activity was reversed on dithiothreitol treatment. However, when the enzyme was preincubated with NADH

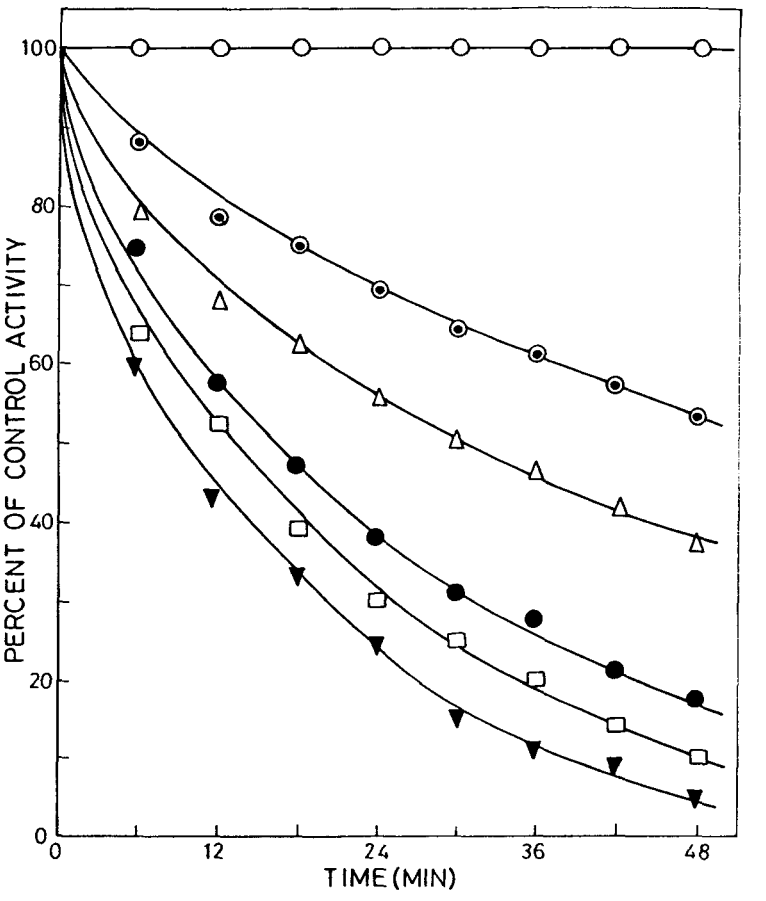

FIGURE 1 Rate of octopine dehydrogenase inactivation by *o*-phthalaldehyde. The enzyme (4 μM) was incubated with the following different concentrations of *o*-phthalaldehyde: (O), o mM; (⊙), 20 μM; (△), 40 μM; (●), 60 μM; (□), 80 μM; (▼), 100 μM. The incubation was carried out in 0.1 M potassium phosphate buffer (pH 7.0) at 25°C. Aliquots were withdrawn at indicated time intervals and residual activity was determined as described in the experimental procedure.

followed by the addition of PCMS and *o*-phthalaldehyde, the resultant inhibition obtained was only due to *o*-phthalaldehyde (Figure 3). This indicated that *o*-phthalaldehyde does not bind at the NADH binding site. The data obtained from the protection studies showed that none of the other substrates except L-arginine protected the enzyme inactivation against *o*-phthalaldehyde (Table I). All this evidence demonstrated that binding of *o*-phthalaldehyde does not take place at the NADH binding domain which is the single catalytic center initially present in octopine dehydrogenase.

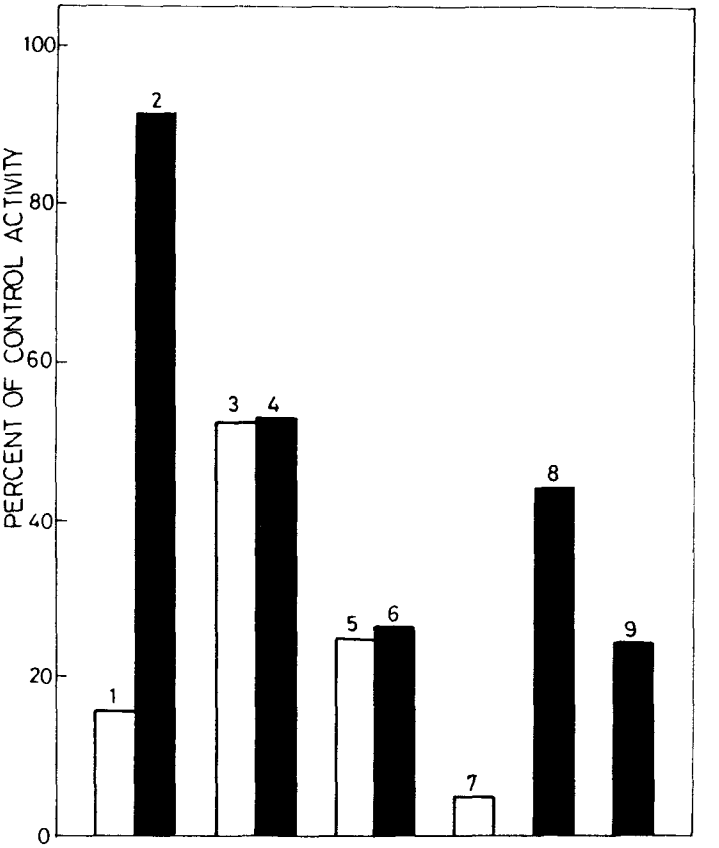

FIGURE 2 Double inhibition of octopine dehydrogenase by PCMS and o-phthalaldehyde. The enzyme (4 μM) was incubated with inhibitors in 0.1 M potassium phosphate buffer (pH 7.0) at 25°C for different time intervals as follows: 20 μM PCMS for 8 min (bar 1,2). 100 μM o-phthalaldhyde for 8 min (bar 3,4) or for 16 min (bar 5,6), 20 μM PCMS for 8 min followed by 100 μM o-phthalaldehyde for 8 min (bar 7,8) or vice versa (bar 9). After the above treatments aliquots were assayed for the enzyme activity (open bars). The remaining incubation mixtures were treated with 20 mM cysteine and 5 mM β-mercaptoethanol for 5 min before activity measurements were carried out (solid bars). Control sets were run under similar conditions in the absence of inhibitors.

The loss of octopine dehydrogenase activity by o-phthalaldehyde was prevented in the presence of α-amino and -SH group containing reagents as shown in Figure 4. Fluorescence emission spectra of the enzyme incubated with 1 mM valine followed by the addition of o-phthalaldehyde did show any characteristic emission for isoindole derivative formation (Figure 5, curve 1). The data provides evidence that an amino group may form a complex with o-phthalaldehyde which prevents it from binding with the enzyme molecule. It has been reported earlier that o-phthalaldehyde can alone form a non-fluorescent complex with amino group-containing reagents.[14,23,24] When the enzyme was incubated with β-mercaptoethanol a fluorescence emission maximum

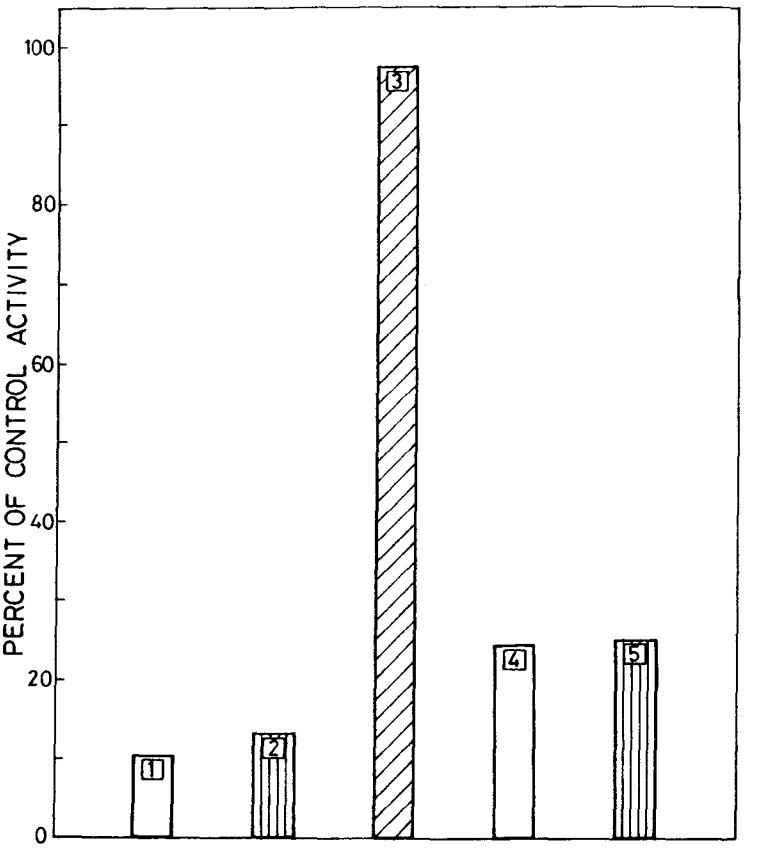

FIGURE 3 Effect on octopine dehydrogenase inactivation by PCMS and *o*-phthalaldehyde using dithiothreitol as activity reversal reagent. The enzyme (4 μM) was incubated with 20 μM PCMS for 20 min (bar 1), 24 mM L-arginine followed by 100 μM *o*-phthalaldehyde for 25 min and then with 20 μM PCMS for 20 min (bar 2). This mixture was treatment with 40 mM dithiothreitol for 2 min (bar 3). In other sets 100 μM *o*-phthalaldehyde was incubated with enzyme for 25 min (bar 4), also, 0.6 mM NADH-treated enzyme was incubated with 20 μM PCMS for 20 min followed by *o*-phthalaldehyde for 25 min (bar 5). At the end of the indicated treatments the residual activity was measured as described in experimental procedure.

was obtained in the presence of *o*-phthalaldehyde which was characteristic of isoindole derivative formation (Figure 5 curve 2). This establishes that ϵ-NH_2 group of octopine dehydrogenase is participating in the *o*-phthalaldehyde reaction. In order to ascertain the accessibility of ϵ-NH_2 group of lysine, pyridoxal 5′-phosphate (PLP) and 5′-*p*-fluorosulfonylbenzoyl adenosine were incubated with the enzyme which showed no loss of enzyme activity. But, a typical absorption and fluorescence peak were obtained in the PLP – treated enzyme at 325 nm and 395 nm respectively as shown in Figure 6 (A,B). These results clearly demonstrated that although lysine is accessible to binding with ϵ-amino reagent it is non-essential for the enzyme catalysis. Earlier studies of

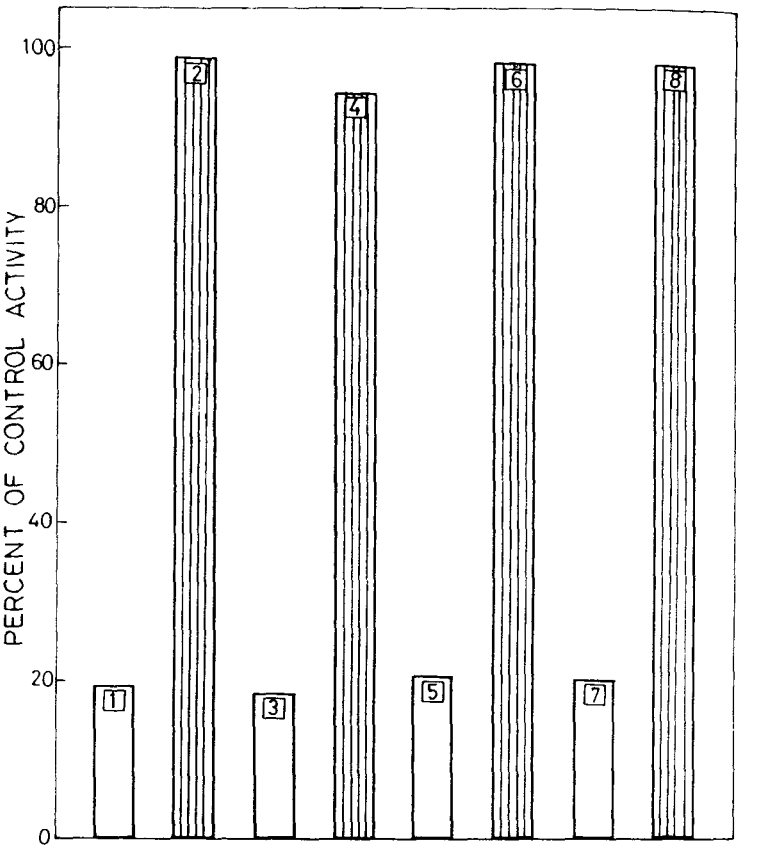

FIGURE 4 Protection of octopine dehydrogenase inactivation against o-phthalaldehyde by amino- and thiol group containing reagents. The enzyme (4 μM) was incubated with 100 μM o-phthalaldehyde for 25 min (har 1,3,5,7) in 0.1 M potassium phosphate buffer (pH 7.0) at 25°C. Under similar conditions the enzyme was first incubated with 24 mM L-arginine (bar 2), 24 mM glycine (bar 4), 24 mM valine (bar 6) and 24 mM β-mercaptoethanol (bar 8) prior to the addition of o-phthalaldehyde (100 μM). After the indicated treatments, the residual enzyme activity was determined as described in experimental procedure.

o-phthalaldehyde reaction have shown that in the presence of β-mercaptoethanol, o-phthalaldehyde binds with the ϵ-NH_2 group of proteins.[16] The -SH groups present in the protein are much less reactive than externally added -SH groups in the form of β-mercaptoethanol.[16] Therefore, it can be concluded that o-phthalaldehyde in the presence of either amino or thiol compounds does not affect the octopine dehydrogenase activity and the inactivation of enzyme by o-phthalaldehyde obtained is due to the disruption of the favourable tertiary structure which may hinder the enzyme substrate binding.

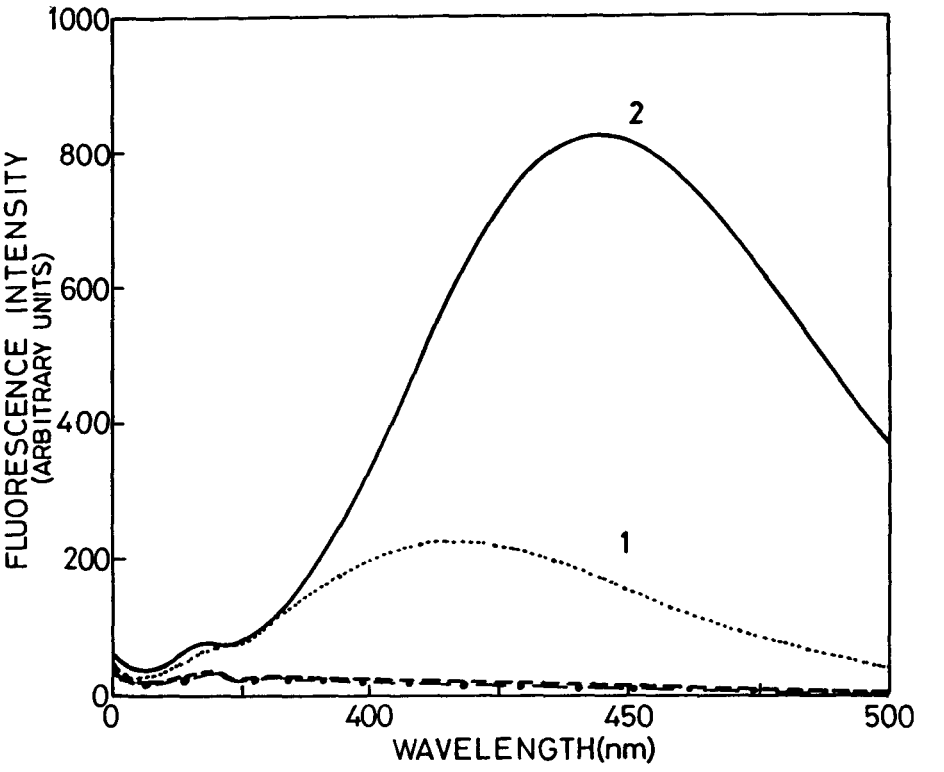

FIGURE 5 Effect of α-amino acid and β-mercaptoethanol on fluorescence emission spectra of octopine dehydrogenase-*o*-phthalaldehyde complex. Octopine dehydrogenase (1 μM) was incubated with 1 mM valine for 5 min in the absence (- - - - -) and in the presence (· · · · · ·) or with 1 mM β-mercaptoethanol for 5 min in the absence (— · — · —) and in the presence (——) of 25 μM *o*-phthalaldehyde for 30 min in 0.1 M potassium phosphate buffer (pH 7.0) at 25°C. Fluorescence emission spectra were recorded with excitation wavelength at 337 nm.

DISCUSSION

The present study has elucidated the nature of *o*-phthalaldehyde reaction with octopine dehydrogenase. *o*-Phthalaldehyde has been used as a fluorescent affinity reagent which readily modifies the functional -SH and ϵ-NH_2 groups at the catalytic center in many enzymes.[7,8,9,10,11] However, present studies clearly demonstrated that *o*-phthalaldehyde does not react with essential cysteine located at the active center but reacts with a cysteine and lysine, both of which are non-essential and in close proximity to each other yet away from the active site region. The crosslinking of these

TABLE 1
Effect of substrates against octopine dehydrogenase inactivation by o-phthalaldehyde.

SUBSTRATES PREINCUBATED WITH OCTOPINE DEHYDROGENASE	PERCENT OF CONTROL ACTIVITY
None	25
NADH (0.6 mM)	23
NADH (1.4 mM)	24
Pyruvate (12 mM)	23
Pyruvate (28 mM)	26
L-arginine (24 mM)	100
L-arginine (56 mM)	99
L-arginine (12 mM) + pyruvate (6 mM)	86
L-arginine (28 mM) + pyruvate (14 mM)	91

The enzyme (4 μM) was preincubated with different substrates at the indicated concentrations in 0.1 M potassium phosphate buffer (pH 7.0) for 5 min. This mixture was then allowed to react with 100 μM o-phthalaldehyde for 25 min and then the remaining activity was measured as described in the experimental procedure.

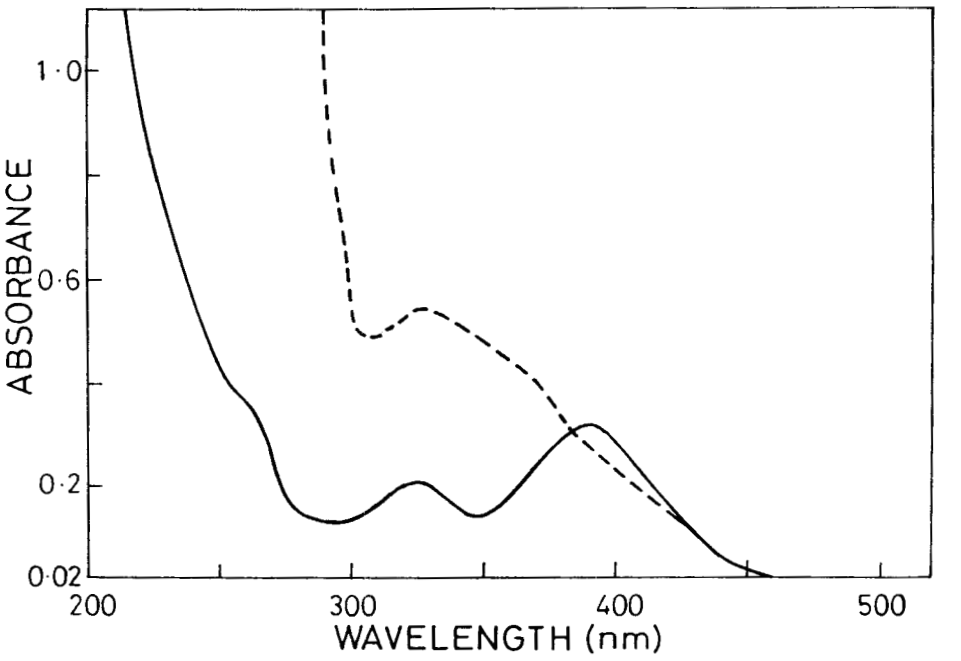

FIGURE 6(A)

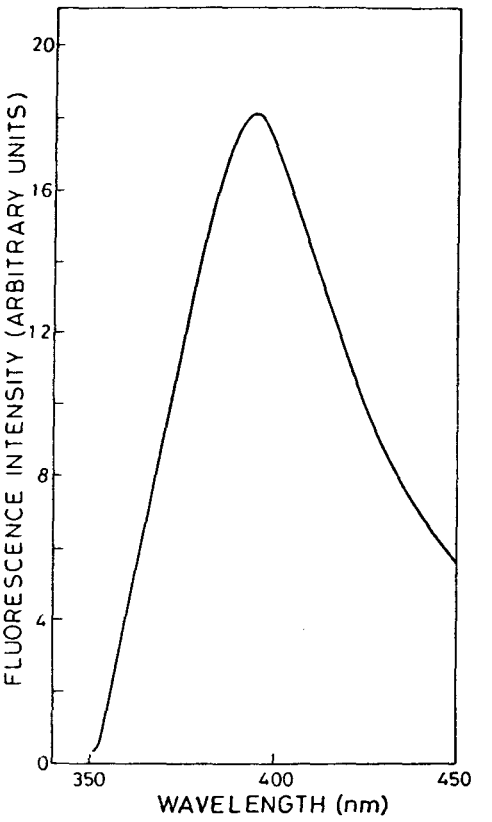

FIGURE 6 Absorption and fluorescence emission spectra of octopine dehydrogenase-PLP complex. (A) Octopine dehydrogenase (0.4 μM) was treated with 53 μM PLP for 20 min in 0.1 M potassium phosphate buffer, pH 7.0, then the absorption spectrum (——) was recorded. This enzyme-PLP product was reduced with 10 mM $NaBH_4$ and dialyzed for 18 h against the same buffer, then the absorption spectrum (- - - -) of the reduced complex was again recorded. (B) Similarly emission spectrum of the reduced enzyme-PLP complex was recorded with excitation wavelength at 325 nm.

residues results in conformational changes leading to the loss of catalytic activity of octopine dehydrogenase.

o-Phthalaldehyde is not an effective inhibitor in the presence of amino acids indicating that the amino group may bind with *o*-phthalaldehyde resulting in the formation of a nonfluorescent adduct as also observed in the reaction of amino compounds with *o*-phthalaldehyde where a cyclic complex is formed.[14,23,24] It was found that α and ϵ-amino groups reacted at different rates, with ϵ-amino group reagent reacting 10 times faster than the α-amino group containing reagent.[13] Moreover, when *o*-phthalaldehyde-amine complex is mixed together with a thiol reagent, fluorescence yield for the reaction is much lower.[14,15] A differential nature in the

o-phthalaldehyde reaction with octopine dehydrogenase was observed in the presence of β-mercaptoethanol. Here, o-phthalaldehyde reacts with the ϵ-NH_2 of the lysine present in the enzyme molecule forming a characteristic isoindole product with β-mercaptoethanol. No loss of enzyme activity was obtained as the ϵ-NH_2 group of lysine in octopine dehydrogenase was found to non-essential but accessible, as also indicated by spectral data for pyridoxal 5′-phosphate binding experiments with the ϵ-amino group of lysine of the enzyme. Similar results were obtained with malate dehydrogenase where the concentration of o-phthalaldehyde used for enzyme inactivation was much lower as compared to pyridoxal 5′-phosphate (data not shown). Therefore, it was observed that in octopine dehydrogenase o-phthalaldehyde reacts with the cysteine which is present near the lysine and not with the cysteine which is located at the catalytic site. The crosslinking of these residues which are in close proximity leads to isoindole derivative formation. This results in a loss of activity due to a change in the conformation of the enzyme molecule.

Thus, from the above results it is evident that the presence of external amino or thiol group donors may alter the normal course of o-phthalaldehyde reaction with the enzymes. It is worth mentioning that enzymes having amino- or thiol-containing compounds as substrates may interfere with the enzyme o-phthalaldehyde reaction resulting in protection. Therefore, precautions must be taken in interpreting the results of chemical modification studies using a o-phthalaldehyde probe in enzymes, where substrates have amino or thiol groups.

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References

1. Simons, S.S., Jr. and Jhonson, D.F. (1976) *J. Amer. Chem. Soc.*, **98**, 7098.
2. Simons, S.S., Jr. and Jhonson, D.F. (1978) *J. Org. Chem.*, **43**, 2886.
3. Hare, P.E. (1977) *Meth. Enzymol.*, **47**, 3.
4. Simons, S.S., Jr., Thomson, E.B. and Jhonson, D.F. (1979) *Biochemistry*, **18**, 4915.
5. Roth, M. and Hampal, A. (1973) *J. Chromatogr.*, **83**, 353.
6. Benson, J.R. and Hare, P.E. (1975) *Proc. Nat. Acad. Sci. USA*, **72**, 619.
7. Palczewski, K., Hargrave, P.A. and Kochman, M. (1983) *Eur. J. Biochem.*, **139**, 429.
8. Puri, R.N., Bhatnagar, D. and Roskoski, R. Jr. (1985) *Biochemistry*, **24**, 6508.
9. Chen, C.-Y., Emig, F.A., Scharmm, V.L. and Ash, D.E. (1979) *J. Biol. Chem.*, **266**, 16645.
10. Kobayashi, M., Miura, M., Watanabe, T. and Ichishima, E. (1991) *Arch. Biohem. Biophys.*, **289**, 350.
11. Sheikh, S. and Katiyar, S.S. (1992) *Biochem. Int.*, **27**, 517.
12. Lee, K.S. and Drescher, D.G. (1978) *Int. J. Biochem.*, **9**, 457.
13. Chen, R.F., Scott, C. and Trepman E. (1979) *Biochim. Biophys. Acta*, **576**, 440.
14. Trepman, E. and Chen, R.F. (1980) *Arch Biochem. Biophys.*, **204**, 524.
15. Roth, M. (1971) *Anal. Chem.*, **43**, 880.
16. Goodno, C.C., Swaisgood, H.E. and Catigani, G.L. (1981) *Anal. Biochem.*, **115**, 203.
17. Olomucki, A. (1981) *Biochem. Soc. Trans.*, 278.
18. Sheikh, S. and Katiyar, S.S. (1993) *Biochem. Biophys. Acta*, **1202**, 251.

19. Sheikh, S. and Katiyar, S.S. (1993) *Biochem. Mol. Biol. Int.*, **29**, 719.
20. Doublet, M.0. and Olomucki, A. (1975) *Eur. J. Biochem.*, **59**, 175.
21. Schrimsher, J.L. and Taylor, K.B. (1984) *Biohemistry*, **23**, 1348.
22. Katiyar, S.S. and Porter, J.W. (1982) *Biochem. Biophys. Res. Commun.*, **107**, 1219.
23. Dominh, T., Jhonson, A.L., Jones, J.E. and Senise, P.P. Jr. (1977) *J. Org. Chem.*, **42**, 4217.
24. Nan'ya, S., Tange, T. and Maekawa, E. (1985) *J. Heterocyclic Chem.*, **22**, 449.