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β-CYCLOPIAZONATE OXIDOCYCLASE FROM *PENICILLIUM CYCLOPIUM*

III. PRELIMINARY STUDIES ON THE MECHANISM OF ACTION

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SUMMARY

1. The isolation of β-cyclopiazonate oxidocyclase from the culture medium of *Penicillium cyclopium* is described.

2. Initial velocity studies using variable concentrations of one substrate and changing fixed levels of the other substrate gave rise to parallel double reciprocal plots.

3. α-Cyclopiazonic acid shows competitive inhibition with respect to β-cyclopiazonic acid.

4. Spectrophotometric titrations of the enzyme with substrate under aerobic conditions shows that the reaction goes to completion to yield oxidized enzyme *plus* α-cyclopiazonic acid.

5. Spectrophotometric titrations of the enzyme with a series of indole derivatives as substrate analogues shows the involvement of the tetramic acid ring in binding of the substrate to the enzyme, whereas the indole ring seems to be responsible for changes in the flavin spectrum.

6. Low temperature studies shows that the reduction of the enzyme by β-cyclopiazonic acid is at least biphasic.

INTRODUCTION

The isolation, purification and characterization of the β-cyclopiazonate oxidocyclase isoenzymes has recently been reported[1,2]. The enzyme catalyzes the conversion of β- to α-cyclopiazonic acid in the presence of a suitable electron acceptor.

Abbreviations: CPA, cyclopiazonic acid; DCIP, 2,6-dichlorophenolindophenol.

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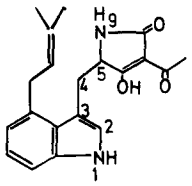
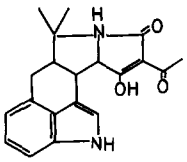


β-cycloplazonic acid



α-cyclopiazonic acid

This enzyme is of considerable interest for a study of complex formation between the enzyme-bound flavin and the indole ring which occurs in both β- and α-cyclopiazonic acid.

The possible importance of flavin–indole complexes in flavoenzyme catalysis and other biological processes has been pointed out by several workers[3–6]. There is at present some uncertainty as to whether flavin–indole complexes are of the charge transfer type. Criteria for the recognition of charge transfer complexes had been suggested by Mulliken[7] and were discussed more recently by Kossower[8,9]. The formation of a charge transfer complex should give rise to a new absorption band, called a charge transfer band, which must be sensitive to the nature of substituents on either the donor or acceptor and to the polarity of the solvent.

Several authors have come to the conclusion that charge transfer forces were indeed involved in the formation of flavin–indole complexes[10–12]. Ehrenberg and Hemmerich[13] discount the involvement of charge transfer forces in the formation of complexes between heteroaromatic amino acids and flavins. Difficulties inherent to establishing the charge transfer nature of flavin–indole complexes were discussed by Tollin[14] and Weber[15].

In this paper the bisubstrate kinetics of β-cyclopiazonate oxidocyclase are described. The essential irreversibility of the enzymatic reaction is demonstrated. Experimental data relating to the nature of flavin–indole complexes are presented.

MATERIALS AND METHODS

Chemicals

β- and α-cyclopiazonic acid were obtained as described[16,17]. Dihydro-β-cyclopiazonic acid, tetramic acid of tryptophan and 3-acetyl trimethylene tetramic acid were gifts from Prof. C. W. Holzapfel, to whom we would like to express our gratitude. L-Tryptophan, indole acetic acid and indole propionic acid were obtained from E. Merck, Darmstadt, Germany as chromatographically homogeneous products.

Cytochrome *c* was a product of Seravac Laboratories, Epping Industria, Cape Town.

Enzyme assay methods

Enzyme assays were performed as described by Schabort *et al.*[1] with the following minor modifications: the standard assay mixture contained 0.05 M sodium phosphate buffer, pH 6.8, instead of sodium maleate and was 1.67% (v/v) with respect to methanol. The temperature was regulated at 30 °C by the circulation of thermostated water through the constant temperature cell housing of an Aminco-Chance dual wavelength split-beam spectrophotometer.

Enzyme activities were calculated from the molar absorbance coefficient for 2,6-dichlorophenolindophenol (DCIP) which is given as 21 000[18–20].

Apparent Michaelis constants for DCIP and β-cyclopiazonic acid were reported by Schabort and Potgieter[2]. Because of the low values for both substrates, it was found advantageous to use cytochrome *c* as a terminal electron acceptor and DCIP as an intermediate for the determination of initial velocities in kinetic studies, in order to enhance the absolute change in absorbance during the reaction. In such cases cytochrome *c* was included in the reaction mixtures at a constant high level. The reaction rate was estimated by measuring the appearance of absorbance at 550 nm and using a molar absorbance index difference of 19 700 $M^{-1} \cdot cm^{-1}$ (see ref. 21) in subsequent calculations.

In view of the low Michaelis constant for DCIP it is also imperative to take precautions against contamination by substances which could serve as intermediate electron acceptors to cytochrome *c*, in order to avoid artefacts. All glassware and cuvettes were rinsed repetitively with chromic acid and deionized water.

Enzyme preparation

The β-cyclopiazonate oxidocyclase isoenzymes were isolated and purified from the mycelium of *Penicillium cyclopium* Westling as described[1] with the following minor modification: the concentration of the enzyme from dilute solutions was achieved by ultrafiltration on an Amicon ultrafiltration apparatus using PM 30 filters. When the enzyme was extracted from the culture medium the "Extraction. Step 1" in the procedure was omitted.

The work described in this paper was performed on an isoenzyme which was isolated from the culture medium of *P. cyclopium* and which corresponded in its elution properties on CM-Sephadex chromatograms to the isoenzyme III obtained by Schabort *et al.*[1]. This isoenzyme had a specific activity of 103 nmoles/min per mg enzyme protein and was found to be stable for at least a year if kept in 20% ethylene glycol (v/v) at −10 °C.

Enzyme protein was determined by the Lowry method as given by Bailey[22].

Initial velocity and inhibition kinetic studies

The required concentrations of the substrates and inhibitor in reaction mixtures were obtained by the addition of these components in small quantities from concentrated stock solutions by means of Terumo microsyringes. The volume changes produced by these additions amounted to less than 1% in any one set of kinetic experiments for a double reciprocal plot and were not taken into account in subsequent calculations. The points shown in double reciprocal plots are the averages of at least two independent determinations.

Spectrophotometric titrations

The effects of β- and α-cyclopiazonic acid and various substrate analogues on the visible spectrum of the oxidized enzyme were studied at room temperature (approx. 22 °C) by recording absorption spectra before and after the addition of various amounts of the substrate analogue from concentrated stock solutions in methanol to the enzyme. The enzyme was contained in 2.5 ml of a mixture comprising 0.05 M sodium phosphate, pH 6.8, and ethylene glycol in the ratio 19:1. Spectra were re-

corded by means of an Aminco Chance dual wavelength split-beam spectrophotometer using the 0 to 0.2 absorbance scale expansion on the recorder and were corrected for volume changes due to the addition of the substrate analogue, which were small in comparison to the total volume. The enzyme concentration was estimated by using a value of 11 300 for the molar extinction coefficient of the enzyme-bound flavin at 450 nm[23].

Low temperature studies on the effect of β-cyclopiazonic acid on the absorbance at 450 nm of the enzyme as a function of time

The cuvette lifters in the cell housing of the Aminco Chance spectrophotometer were modified to allow the passage of nitrogen gas from a nitrogen gas cylinder through a liquid nitrogen reservoir to affect cooling of the cuvettes. This technique also served to avoid condensation on the walls of the cuvettes. The temperature of the reaction mixture was measured prior to and after the completion of the reaction. The enzyme was contained in 2.5 ml of a mixture of 0.05 M sodium phosphate, pH 6.8, and ethylene glycol in the ratio 8:2. The substrate was added in the desired amount by means of a Terumo microsyringe after temperature equilibration of the enzyme solution and recording was commenced 5 s later. The absorbance change at 450 nm was followed until the completion of the reaction.

RESULTS AND DISCUSSION

Enzyme purification

It was found that the enzyme was released in substantial amounts into the culture medium of the fungus on the fourth day of incubation and that the activity declines slowly during the following days (Table I). Improved yields of enzyme could be obtained by purification of the enzyme from the culture medium. It was found that the relative amounts of the isoenzymes which were obtained from the culture medium were not constant for different batches of culture medium. In some cases the majority of the enzyme was eluted in 0.005 M sodium phosphate buffer, pH 6.8, from CM-Sephadex C-50 and only trace amounts of the other isoenzymes could be found.

A further resolution of the first component to be eluted from a CM-Sephadex

TABLE I

THE PRODUCTION OF β-CYCLOPIAZONATE OXIDOCYCLASE BY *Penicillium cyclopium* AS A FUNCTION OF TIME

*Incubation time** *(h)*	*Weight of wet mycelia (g)*	*Volume of medium (ml)*	*Activity***
70	10	165	—
96	10	165	17.9
120	10	165	17.28
192	15	160	13.9

* Time at which the incubation mixtures were placed in a deep freeze at −20 °C.

** Arbitrary units of activity: decrease in absorbance at 600 nm of 0.001/min per ml enzyme solution[1].

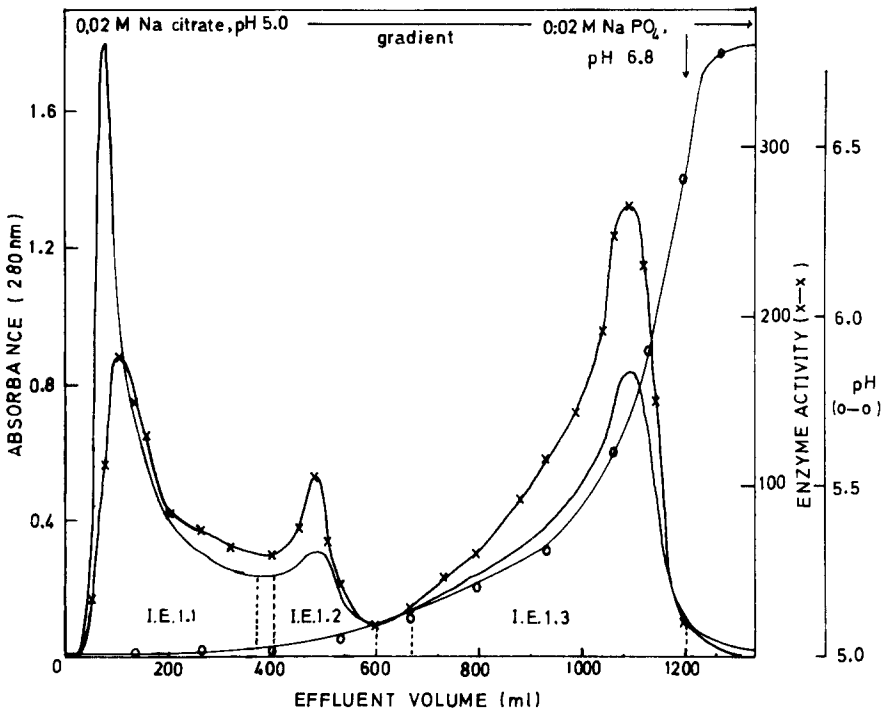


Fig. 1. Further purification of an impure fraction of β-cyclopiazonate oxidocyclase by CM-Sephadex chromatography. Fractions were sampled for protein. ($A_{280\ nm}$, unbroken line), enzyme activity (in arbitrary units as defined in the footnote to Table I, ×—×) and pH (○—○). Column dimensions: 2.1 cm × 40 cm. Flow rate: 25 ml/h. I.E., isoenzyme.

C-50 column in 0.005 M sodium phosphate, pH 6.8, could be obtained by means of a pH gradient as is shown in Fig. 1. The three isoenzymes which were obtained in this manner were designated isoenzymes 1.1, 1.2 and 1.3 and had specific activities of 57.0, 65.8 and 98.5 nmoles/min per mg enzyme protein, respectively. These isoenzymes had anomalous visible spectra, which are probably due to the presence of a contaminant.

Initial velocity studies

The specific activity of the enzyme used in this work corresponds to a turnover rate in moles of substrate per mole enzyme per min of only 4.92 at 30 °C when using DCIP as terminal electron acceptor. Whereas this low turnover makes it possible to study the transient phase of the reaction by conventional spectrophotometry at low temperatures, it does introduce problems in initial velocity studies. The transient phase was evident in initial velocity studies as an initial burst in the disappearance of DCIP or as a lag in the appearance of the difference spectrum at 550 nm when cytochrome *c* was used as the terminal electron acceptor. The interpretation of these phenomena was complicated by slight instrumental instability during the first minute of the reaction at large scale expansions. Attempts to overcome this problem by working at low enzyme concentrations resulted in unrealistically low reaction rates. It was consequently decided to abandon the measurement of "initial velocities" as was suggested by Wong[24] and to measure the slope of reaction

curves after 2 min of reaction time. The error introduced by this procedure was probably less serious when cytochrome *c* was used as a terminal electron acceptor, since DCIP is then constantly regenerated and maintains a reasonably constant level.

The relative efficiency of electron transfer to cytochrome *c* without and with DCIP as an intermediate was determined using reaction mixtures containing $3.76 \cdot 10^{-5}$ M β-cyclopiazonic acid, $4.49 \cdot 10^{-5}$ M cytochrome *c* and $1.4 \cdot 10^{-7}$ M enzyme in 0.05 M phosphate buffer, pH 6.8. The rate of the reaction was measured before and after the addition of DCIP to give a concentration of $4.46 \cdot 10^{-6}$ M DCIP in the reaction mixture. Under these conditions the rate of reduction of cytochrome *c* in the absence of DCIP was only 3.8% of the rate with DCIP as an intermediate, and is

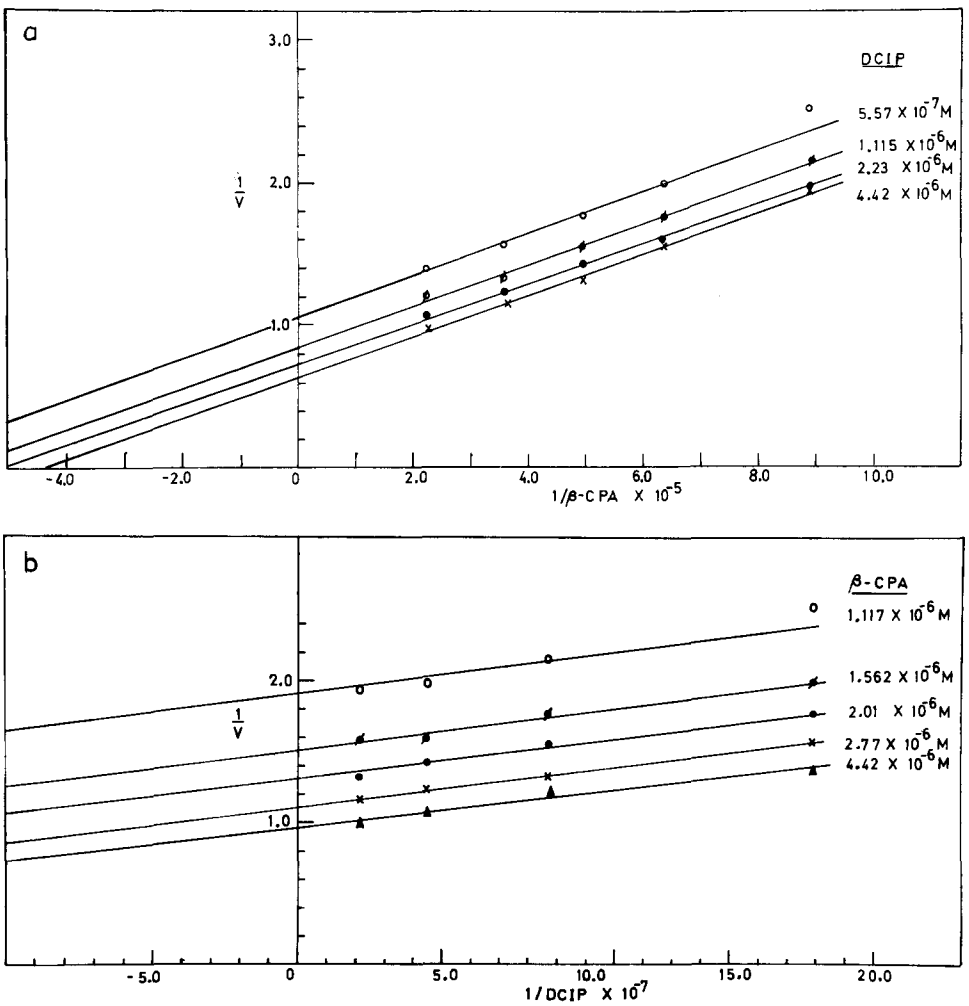


Fig. 2. (a) Primary double reciprocal plots showing the dependence of reaction velocity, expressed in units of 100 nmoles·l^{-1}·min^{-1}, on β-cyclopiazonic acid (β-CPA) concentration at various fixed levels of DCIP. Cytochrome *c* was maintained at a constant level of $5.64 \cdot 10^{-5}$ M and the enzyme concentration was $2.66 \cdot 10^{-8}$ M. Determinations were performed at 30 °C in 0.05 M sodium phosphate buffer, pH 6.8. (b) Primary double reciprocal plots of the data in (a) showing the dependence of reaction velocity on DCIP concentration at various fixed levels of β-cyclopiazonic acid.

possibly dependent upon the production of H_2O_2 (ref. 25) since atmospheric oxygen can function as a weak electron acceptor.

Primary double reciprocal plots showing the relationship between β-cyclopiazonic acid and DCIP as changing fixed and as variable substrates are shown in the Figs 2a and 2b. A series of parallel straight lines were obtained. Secondary plots of intercepts on the vertical axis are shown in Fig. 3.

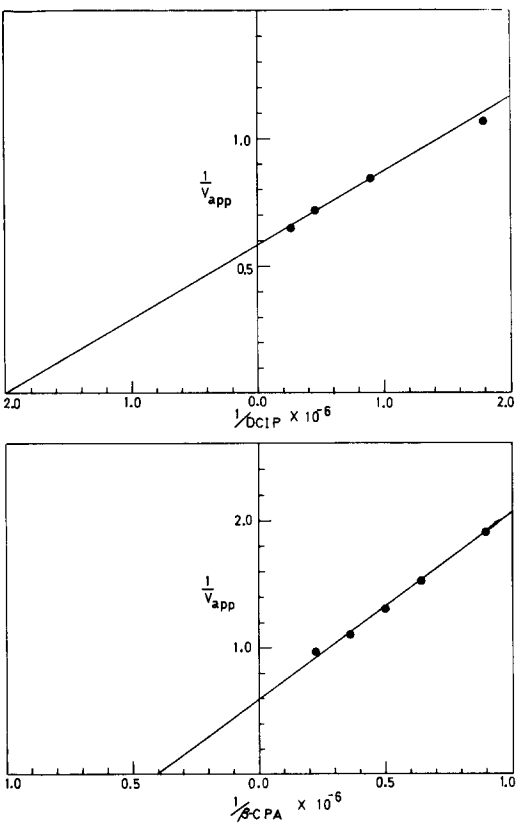


Fig. 3. Replots of the intercepts on the vertical axes of Figs 2a and 2b. CPA, cyclopiazonic acid.

Similar results were obtained when DCIP was used as the sole electron acceptor. These results are indicative of a Ping-Pong Bi-Bi mechanism and $K_{\beta\text{-CPA}}$ and K_{DCIP} (CPA, cyclopiazonic acid) in the reciprocal rate equation:

$$\frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{K_{\beta\text{-CPA}}}{\beta\text{-CPA}} + \frac{K_{\text{DCIP}}}{\text{DCIP}}\right)$$

were evaluated. $K_{\beta\text{-CPA}} = 2.5 \cdot 10^{-6}$ M. $K_{\text{DCIP}} = 0.5 \cdot 10^{-6}$ M.

It was pointed out by Dervartanian *et al.*[26] that double reciprocal plots having an intersecting initial velocity pattern may appear parallel if the constant term in the initial velocity rate equation, written in the notation of Cleland[27], $v = V_1\text{AB}/K_{ia}K_b + K_b\text{A} + K_a\text{B} + \text{AB}$ is small.

However, product inhibition studies may also be used to validate initial velocity patterns[27].

Kinetics of the inhibition by α-cyclopiazonic acid

Double reciprocal plots of the inhibition by α-cyclopiazonic acid using β-cyclopiazonic acid as variable substrate were obtained at two different unsaturating levels of DCIP. In both cases the inhibition appeared to be competitive with respect to β-cyclopiazonic acid. One of these inhibition patterns is shown in Fig. 4.

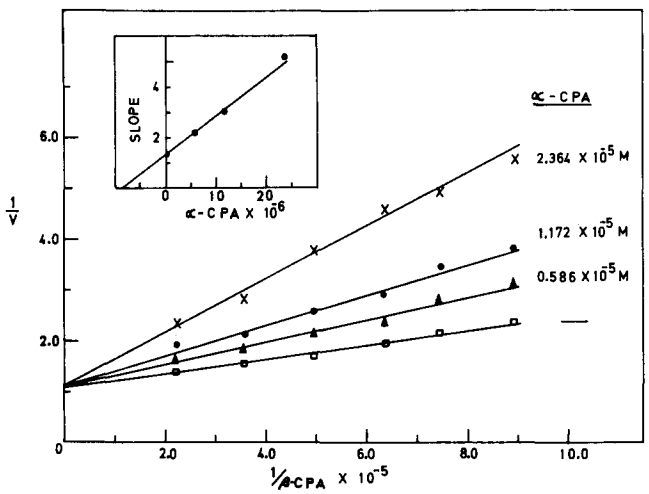


Fig. 4. Primary double reciprocal plots showing the effect of various fixed levels of α-cyclopiazonic acid on reaction velocity, expressed in units of 100 nmoles·l^{-1}·min^{-1}, with β-cyclopiazonic acid as variable substrate. DCIP was maintained at a constant level of 1.76·10^{-6} M and determinations were performed at 30 °C in 0.05 M sodium phosphate buffer, pH 6.8. The enzyme concentration was 2.00·10^{-8} M. A secondary slopes replot is shown in the inset. CPA, cyclopiazonic acid.

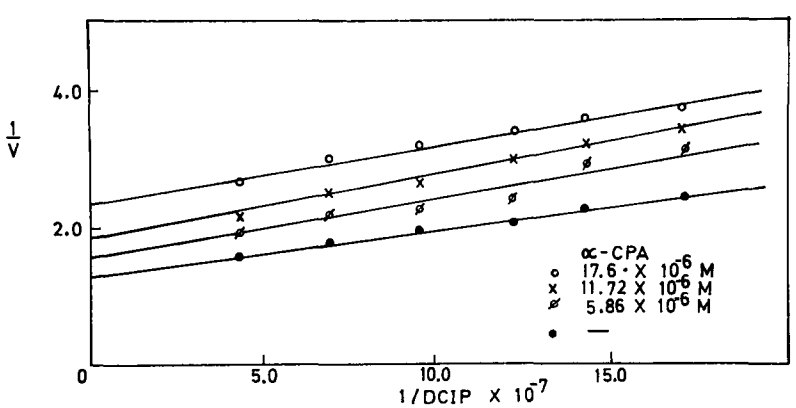


Fig. 5. Primary double reciprocal plots showing the effect of α-cyclopiazonic acid on reaction velocity expressed in units of 100 nmoles·l^{-1}·min^{-1} with DCIP as variable substrate. The β-cyclopiazonic acid concentration was maintained at a constant level of 2.2·10^{-6} M and the enzyme concentration was 2.00·10^{-8} M. All determinations were performed at 30 °C in 0.05 M sodium phosphate buffer, pH 6.8. CPA, cyclopiazonic acid.

The effect of α-cyclopiazonic acid on double reciprocal plots uisng DCIP as a variable substrate is shown in Fig. 5.

Because of the autooxidizability of reduced DCIP[28], product inhibition studies using a second substrate could not be performed.

The inhibition kinetics of α-cyclopiazonic acid are entirely contrary to that expected in a Ping-Pong Bi-Bi mechanism, but may also be explained in terms of the formation of a dead-end inhibitor complex between the oxidized enzyme and α-cyclopiazonic acid, if the rate constant for the association of the reduced enzyme and α-cyclopiazonic acid is small.

Evidence in support of the latter possibility was obtained by means of spectrophotometric studies on the effect of α-cyclopiazonic acid on the visible spectrum of the oxidized enzyme.

If it is assumed that α-cyclopiazonic acid is a dead-end inhibitor of the enzyme, an inhibition constant, $K_i = 8.60 \cdot 10^{-6}$ M may be calculated.

Spectrophotometric titration of the oxidized enzyme with β- or α-cyclopiazonic acid

Flavoenzymes exhibit marked changes in the visible spectrum upon the addition of substrates. The interpretation of these spectral changes is problematic and has to be correlated with ESR studies in order to come to definite conclusions. The spectral properties of free radical intermediates have, however, been established[29].

The addition of β-cyclopiazonic acid to the enzyme under aerobic conditions resulted in a marked decrease in absorbance of the enzyme in the visible region to yield a spectrum which is presumably characteristic of the steady state for the conversion of β-cyclopiazonic acid to to α-cyclopiazonic acid in the presence of oxygen (Fig. 6).

The curves which were eventually obtained when no further changes in the spectra occurred were dependent on the amount of β-cyclopiazonic acid which was initially added and exhibit four isosbestic points which indicates the presence of only

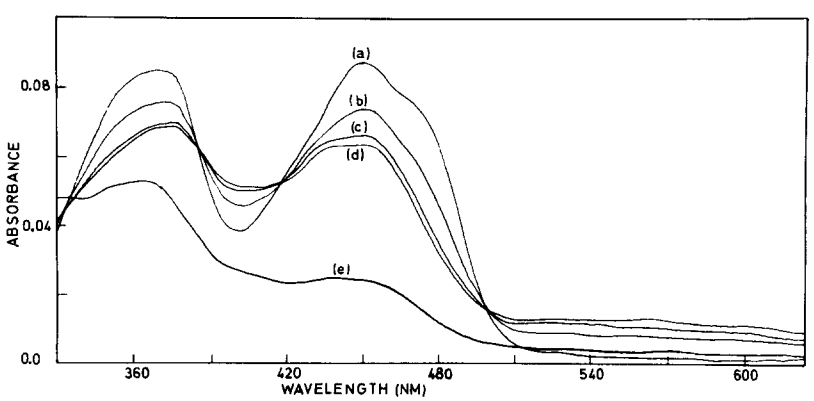
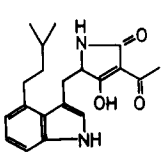


Fig. 6. Aerobic spectrophotometric titration of β-cyclopiazonate oxidocyclase with β-cyclopiazonic acid. Curves were recorded when no further changes in absorbance took place, after the addition of β-cyclopiazonic acid to give the following concentrations: (a) oxidized enzyme with no additions; (b) $1.3 \cdot 10^{-5}$ M β-cyclopiazonic acid; (c) $3.9 \cdot 10^{-5}$ M β-cyclopiazonic acid; (d) $6.5 \cdot 10^{-5}$ M β-cyclopiazonic acid; and (e) when the absorbance at 450 nm had reached a minimum value after the addition of β-cyclopiazonic acid to (c) to obtain (d).

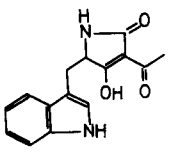
two species. Curves which were quantitatively and qualitatively similar to that shown in Fig. 6 were obtained when the enzyme was titrated with α-cyclopiazonic acid. It is evident that the curves shown in Fig. 6 result from the interaction of the procuct, α-cyclopiazonic acid, with the oxidized enzyme. Moreover, the curves in Fig. 6 demonstrate the essential irreversibility of the oxidocyclization reaction. There are no indications of any other enzyme species than the oxidized enzyme and an oxidized enzyme–α-cyclopiazonic acid complex. The α-cyclopiazonic acid could be removed from the enzyme by gel chromatography on Sephadex G-25. In cases where β-cyclopiazonic acid was added to the enzyme the low molecular weight component obtained by gel chromatography of the enzyme–α-cyclopiazonic acid complex had an ultraviolet spectrum similar to that of α-cyclopiazonic acid.

Spectrophotometric titration of the oxidized enzyme with substrate analogues

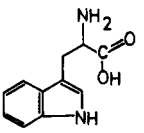
The structures of dihydro-β-cyclopiazonic acid, tetramic acid of tryptophan, L-tryptophan, indole propionic acid, indole acetic acid and 3-acetyl trimethylene tetramic acid are given below.



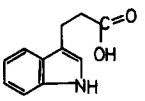
Dihydro-β-cyclopiazonic acid



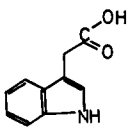
Tetramic acid of tryptophan



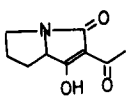
L-Tryptophan



Indole propionic acid



Indole acetic acid



3-Acetyl trimethylene tetramic acid

The effects of these substrate analogues on the visible spectrum of oxidized enzyme were in general similar to that of α-cyclopiazonic acid. All of these compounds, with the exception of 3-acetyl trimethylene tetramic acid, produce a hypochromicity at 450 nm in the flavin spectrum. The exact positions of the isosbestic points and the detailed nature of the curves were, however, different from that obtained in the case of α-cyclopiazonic acid. For purposes of illustration, the titration of the enzyme with L-tryptophan is shown in Fig. 7.

The equations for the determination of association constants from spectrophotometric titrations have been derived by Benesi and Hildebrand[30] and Isenberg and Szent-Györgyi[5]. Because of the low K_D values for some of the enzyme–substrate analogue complexes certain simplifying assumptions in the theory are not possible.

For a dissociating species, $EM \rightleftharpoons E + M$, where E is the enzyme, EM the enzyme–substrate analogue complex and M the substrate analogue

$$K_D = \frac{[E]\,[M]}{[EM]} \qquad (1)$$

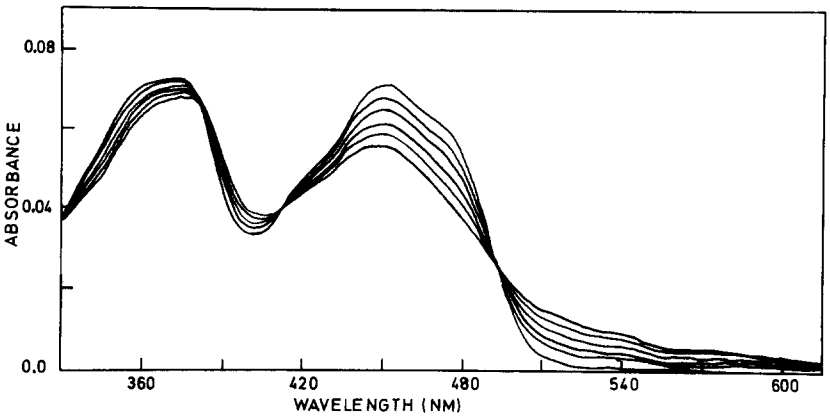


Fig. 7. Aerobic spectrophotometric titration of β-cyclopiazonate oxidocyclase by stepwise addition of L-tryptophan to obtain $1.94 \cdot 10^{-5}$ M, $3.87 \cdot 10^{-5}$ M, $7.73 \cdot 10^{-5}$ M, $13.4 \cdot 10^{-5}$ M and $22.70 \cdot 10^{-5}$ M L-tryptophan.

Assuming that the species EM obeys the Beer–Lambert Law one may write:

$$EM = k' \Delta A_{450} \tag{2}$$

where ΔA_{450} is the difference spectrum between oxidized enzyme and enzyme–substrate analogue complex at 450 nm, and k' is a proportionality constant

$$k' = \frac{1}{\varepsilon_E - \varepsilon_{EM}} \tag{3}$$

ε_E and ε_{EM} are molar extinction coefficients of the enzyme and enzyme–substrate analogue complex, respectively, at 450 nm.

Let E_0 and M_0 equal the initial enzyme and substrate analogue concentrations, respectively, then:

$$K_D = \frac{[E_0 - k' \Delta A_{450}]\,[M_0 - k' \Delta A_{450}]}{k' \Delta A_{450}} \tag{4}$$

which simplifies to

$$\frac{E_0}{\Delta A_{450}} = k' \left(\frac{K_D + E_0 - k' \Delta A_{450}}{M_0} + 1 \right) \tag{5}$$

Curves of $E_0/\Delta A_{450}$ *vs* $1/M_0$ would have a slope of $k'K_D$ at infinite concentration of the substrate analogue and a y-axis intercept equal to k'. The tangent to these curves at infinite substrate analogue concentration should have a x-axis intercept of $-1/K_D$.

When $K_D \gg E_0$ the equation simplifies:

$$\frac{E_0}{\Delta A_{450}} = \frac{k'K_D}{M_0} + k' \tag{6}$$

which corresponds to the form given by Benesi and Hildebrand[30]. Plots of $E_0/\Delta A_{450}$ *vs* $1/M_0$, representing the binding of the different substrate analogues to the enzyme, are shown in Fig. 8 and are drawn linear in cases where K_D is sufficiently larger than E_0. Strong associations between enzyme and sbustrate analogue are manifested in

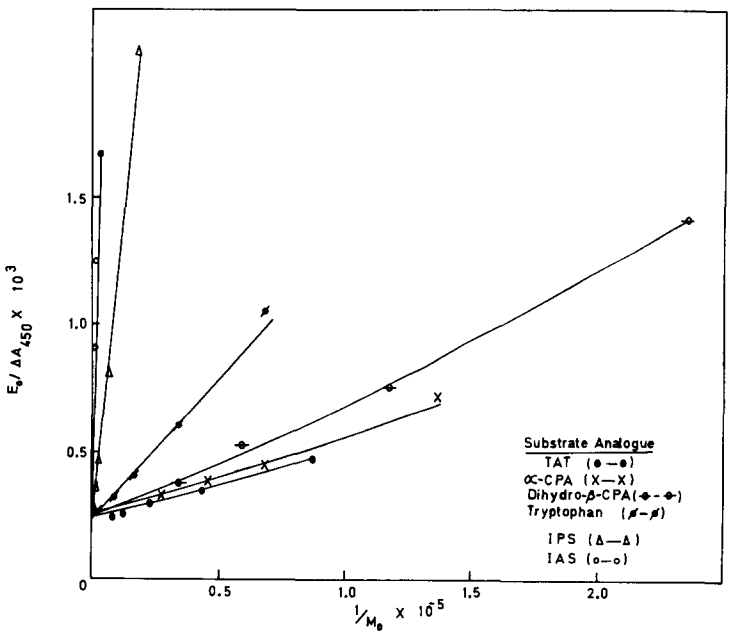


Fig. 8. Plots showing the relationship, given in the text, between the absorbance difference at 450 nm, ΔA_{450} and substrate analogue concentration, M_0 for the different substrate analogues. TAT, tetramic acid of tryptophan; CPA, cyclopiazonic acid; IPS and IAS, indole propionic and acetic acid, respectively.

decreased slopes of the curves. Values of K_D and k' were read directly from the curves and are given in Table II.

It is evident that the substrate analogues fall into three categories: (i) compounds having a tetramic acid ring bind strongly to the enzyme; (ii) the dissociation constant for the enzyme–L-tryptophan complex is intermediate between that of the tetramic acid substituted indoles and (iii) indole derivatives lacking the amino group, which bind weakly to the enzyme.

TABLE II

DISSOCIATION CONSTANTS FOR THE BINDING OF SUBSTRATE ANALOGUES TO β-CYCLOPIAZONATE OXIDOCYCLASE

Substrate analogue	$K_D \cdot 10^5$ *(M)*	$k' \cdot 10^4$ *(M)*
α-Cyclopiazonic acid	0.846	2.6
Tetramic acid of tryptophan	0.88	2.5
Dihydro-β-cyclopiazonic acid	1.34	2.7
L-Tryptophan	4.72	2.5
Indole propionic acid*	50.0	2.0
Indole acetic acid*	86.5	—

* Because of the small absorbance differences and limited solubilities, especially of indole acetic acid, only approximate values could be obtained.

The addition of 3-acetyl trimethylene tetramic acid to the enzyme had no apparent effect on the visible spectrum of the enzyme.

The effect of solvent polarity on the binding of α-cyclopiazonic acid to the enzyme

Shiga *et al.*[31] have reported marked blue shifts in the visible spectrum of D-amino acid oxidase when this enzyme was transferred from a buffered aqueous environment to a buffered ethylene glycol–water mixture (1:1, v/v).

A similar effect on the spectrum of β-cyclopiazonate oxidocylcase is not evident up to a concentration of 28% ethylene glycol. Moreover, the presence of ethylene glycol had no effect on the position of isosbestic points which are obtained by the spectrophotometric titration of the oxidized enzyme with α-cyclopiazonic acid. This finding implies that ethylene glycol does not alter the spectrum of the enzyme or that of the enzyme–α-cyclopiazonic acid complex, and is consistent with the resolution of the flavin absorption band at 450 nm into a band with shoulders at 420 nm and 475 nm[11,32], as in Figs 6 and 7.

The effect of ethylene glycol on the dissociation constant of the enzyme–α-cyclopiazonic acid complex, however, is pronounced, as is evident from Fig. 9.

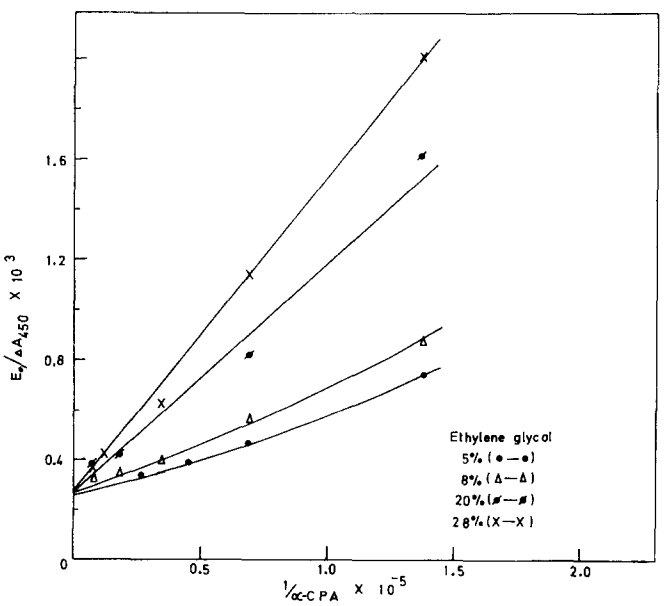


Fig. 9. The effect of ethylene glycol on the binding of α-cyclopiazonic acid (α-CPA) to β-cyclopiazonate oxidocyclase.

Kinetics of the interaction between β-cyclopiazonic acid and the enzyme

Addition of β-cyclopiazonic acid to the enzyme caused marked changes in the absorbance at 450 nm which could be studied by conventional spectrophotometry at low temperatures (Fig. 10). The reduction of the enzyme by β-cyclopiazonic acid is biphasic and appears to consist of a second order reaction which depends on the concentration of β-cyclopiazonic acid, followed by a much slower reaction which depends only on the intermediate which is formed in the initial second order reaction.

The reoxidation of the reduced enzyme by oxygen is a painfully slow process.

Judging by the magnitude of the spectral changes produced, the initial fast reaction may be interpreted as giving rise to the formation of an enzyme–β-cyclopiazonic acid complex, EX, similar to that produced by other indole derivatives, which is converted to the fully reduced enzyme, F, by a slower process.

Attempts to simulate curves, such as are shown in Fig. 10 using six differential equations, which describe the reactions (CPA, cyclopiazonic acid):

$$E + \beta\text{-CPA} \xrightarrow{k_1} EX \xrightarrow{\alpha\text{-CPA}} F \xrightarrow[O_2]{k_3} E + H_2O_2$$

$$E + \alpha\text{-CPA} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} E\text{–}\alpha\text{-CPA complex},$$

in a fourth order Runge Kutta method and using a steepest descent parameter search for the values of the rate constants, yielded poor results. Subsequent analogue computer investigations showed that whereas reasonable fits to the data describing the reductive phase of the reaction could be obtained, the oxidative phase could not be simulated within the framework of this mechanism. In these studies the difference spectra produced by indole derivatives and by dithionite were used to estimate the extinction coefficients of EX and of the reduced enzyme, respectively. It is felt that the postulation of complete reaction mechanisms, when consistency of these mechanisms with the experimental data from turnover experiments had not yet been demonstrated, is premature. An example of the use of computer simulation to substantiate tentative mechanisms may be found in the work of Gibson *et al.*[33].

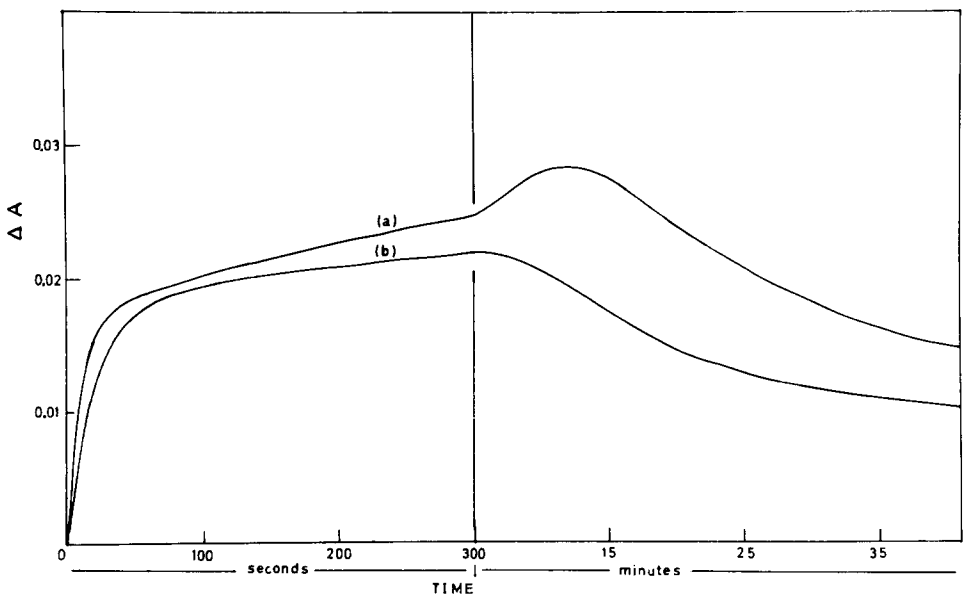


Fig. 10. Changes in absorbance at 450 nm after mixing $4.92 \cdot 10^{-6}$ M β-cyclopiazonate oxidocyclase and (a) $2.23 \cdot 10^{-5}$ M β-cyclopiazonic acid; (b) $1.118 \cdot 10^{-5}$ M β-cyclopiazonic acid (final concentrations). The temperature was maintained at −5.8 °C.

CONCLUSIONS

Initial velocity studies showing seemingly parallel double reciprocal plots are very common for flavoenzymes and do not exclude the possibility of the formation of ternary complexes[26,32]. In the case of β-cyclopiazonate oxidocyclase, the formation of ternary complexes would imply the existence of two binding sites for two rather bulky substrates, β-cyclopiazonic acid and DCIP, in the vicinity of the flavin coenzyme, and the possible involvement of amino acid side chains in the electron transfer between flavin and the two substrates. The involvement of sulfhydryl groups in the mechanisms of catalysis of NADH dehydrogenases[34], thioredoxin reductase[35], lipoyl dehydrogenase[36] and glutathione reductase[37] has been postulated. β-Cyclopiazonate oxidocyclase, however, is not inhibited by *p*-chloromercuribenzoate[2] and no evidence regarding the essentiality of any amino acid side chain for full catalytic activity has thus far been obtained. In view of the model studies on the photoalkylation of flavins by Walker *et al.*[38] and the more complex mechanisms of the external flavoprotein hydroxylases[39,40], it is not certain that objections, on steric grounds, to the formation of ternary complexes are valid.

In view of the essential irreversibility of the conversion of β- to α-cyclopiazonic acid, it seems reasonable to propose that the inhibition by α-cyclopiazonic acid is not true product inhibition, but similar to dead-end inhibition by a substrate analogue and that ternary complexes may not be involved in the catalytic mechanism.

The results of spectrophotometric titrations of the oxidized enzyme with indole derivatives indicate that the tetramic acid residue and especially N-9 is responsible for binding at the enzyme active site. The sensitivity of the dissociation constant of the enzyme–α-cyclopiazonic acid complex to the polarity of the medium (Fig. 9) indicates that the interactions between the tetramic acid ring and the binding site on the enzyme are mainly hydrophobic.

Preliminary studies in this laboratory on the temperature dependence of the dissociation constant for the enzyme–tetramic acid of tryptophan complex indicated that the favourable equilibrium for binding of tetramic acid of tryptophan by the enzyme is largely of an entropic origin, which is compatible with hydrophobic interactions between tetramic acid of tryptophan and the enzyme.

Statements[13] that flavin–indole complex formation is absolutely dependent on water and may be described as ternary complexes of flavin, indole and water do not appear to be consistent with the findings reported here. The partial resolution of the 450 nm band of β-cyclopiazonate oxidocyclase is indicative of a flavin in a nonpolar environment[11,32]. This interpretation is substantiated by the lack of any effect of ethylene glycol on the spectrum of the enzyme or the enzyme–α-cyclopiazonic acid complex[31]. Whereas the dissociation constants of the enzyme–substrate analogue complexes are very sensitive to variations in the nature of the substituent on C-3 of the indole ring, the spectral changes brought about by these substrate analogues are similar and do not yet lend themselves to unequivocal interpretation.

The spectral characteristics of flavin-semiquinones are well documented[29,32]. Whereas α-cyclopiazonic acid, tetramic acid of tryptophan, L-tryptophan and to a lesser extent indole propionic acid show limited signs of long wavelength absorbance, such an effect is virtually absent with dihydro-β-cyclopiazonic acid and indole acetic acid. The limited long wavelength absorbance of the enzyme–substrate analogue com-

plexes appear to rule out the possibility of neutral semiquinones, whereas the hypochromicity in the vicinity of the 380 nm peak is not consistent with an anionic semiquinone species.

The curves resemble those obtained in the titrations of most flavoproteins with their substrates in that semiquinone species seem to be absent.

It may be suggested that the binding of the tetramic acid ring in β-cyclopiazonic acid serves to superimpose the indole ring on the isoalloxazine in a "sandwich"-type of complex as was suggested by Karreman[41] and Isenberg[4], and that the indole ring functions as an electron donor towards the isoalloxazine. Such a mechanism would be analogous to that proposed by Yagi *et al.*[42] for the mechanism of action of D-amino acid oxidase. Complexes of α-cyclopizaonic acid and substrate analogues with the flavin would then correspond to Mulliken's "outer" complexes and only β-cyclopiazonic acid would be able to form an "outer" complex as well as an "inner" complex.

Whatever the case may be, it seems as if the oxidocyclization reaction may be initiated by electron donation from the indole to the isoalloxazine. This suggestion raises interesting questions as to whether electron transfer takes place by two consecutive one-electron transfers or by a two-electron transfer, thus simulating hydride ion transfer, and as to the possible involvement by hyperconjugation of C-4 in electron transfer. At present no adequate answer to these questions can be given.

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