Biochimica et Biophysica Acta, 358 (1974) 126–143

BBA 67263

THE ROLE OF ESSENTIAL HISTIDINES IN THE MECHANISM OF CATALYSIS OF THE FLAVOENZYME, β-CYCLOPIAZONATE OXIDOCYCLASE

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(Received December 17th, 1973)

SUMMARY

1. A study of the pH-dependence of the binding of several indole derivatives to the flavoenzyme, β-cyclopiazonate oxidocyclase, indicates that groups with pK_a values of 6.0 to 7.0 are involved in the binding of these compounds to the active site.

2. It is shown that the first order rate constant for the reduction of the enzyme by enzyme-bound substrate exhibits very little pH dependence.

3. The inactivation of the enzyme by the carbethoxylation of histidyl residues and its reactivation by treatment with hydroxylamine is described.

4. The spectral changes accompanying the binding of the substrate, β-cyclopiazonic acid, to the carbethoxylated enzyme are compared to those which characterize the formation of the enzyme–substrate intermediate complex during catalysis by the native enzyme.

5. The significance of these results in relation to a general mechanism in which histidyl residues function to abstract a proton in flavo enzyme catalysis is briefly discussed.

INTRODUCTION

In spite of significant advances in recent years the functional relationships between the coenzyme and apoenzyme which results in enzymatic activity of the flavoenzymes are still not well understood and the extent to which amino acid side chains on the apoenzyme mediates the reduction of the isoalloxazine ring by the substrate is not clear.

The involvement of sulphydryl groups in the mechanism of catalysis have been

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shown for a number of flavoenzymes [1–4]. The participation of a nucleophilic side chain of the apoenzyme in the mechanism of catalysis of the amino acid oxidases was postulated by Niems et al. [5]. It was subsequently shown that catalysis by amino acid oxidases involved the activation of the amino acid substrate by removal of a proton from the α-carbon atom to allow the reaction to proceed over an enzyme bound amino acid intermediate [6–8].

This mechanism was further substantiated when it was shown that α,β-elimination may occur in favourable cases [9–11] as in the oxidation of β-chloroalanine to pyruvate. Large rate enhancements for the oxidation of carbanionic nitromethane and nitroethane by glucose oxidase and D-amino acid oxidase, respectively, as opposed to the oxidation of the neutral nitroalkanes have been reported [12]. The involvement of a group with an enthalpy of ionization of 7.5 Kcal/mole indicated a histidyl residue as the possible proton acceptor in catalysis by L-amino acid oxidase [13]. Chemical modification studies pointed to the essentiality of a histidyl residue in catalysis by arginine mono-oxygenase [14]. Similar findings were reported for the oxidoreductases which utilize pyridine nucleotides as coenzymes [15–18].

Thus far, however, studies relating to the involvement of groups on the apoenzyme in flavoenzyme catalysis have been limited to a relatively small number of these enzymes. It would therefore be of interest to extend these studies to other lesser well known simple flavoenzymes.

β-Cyclopiazonate oxidocyclase is a flavoenzyme which catalyzes the oxidocyclization of β-cyclopiazonic acid to α-cyclopianozic acid in the presence of atmospheric O_2 or artificial electron acceptors [19,20]. β-Cyclopiazonic acid is the only known substrate of this enzyme. The enzyme, however, forms spectrophotometrically observable complexes with a number of substrate analogues which are invariably indole derivatives [21]. In contrast to many other flavoenzymes such as succinate dehydrogenase [22], D-amino acid oxidase [23–25], salicylate hydroxylase [26] and lactate oxidase [27] which appear to have a strict charge requirement in as much as a carboxyl or other negatively charged group seems to be a prerequisite for binding of compounds at the active site, β-cyclopiazonate oxidocyclase forms complexes with both positively and negatively charged indole derivatives [21, 28].

The pH-dependence of the binding of these indole derivatives and of the first order rate constant for the reduction of the flavocoenzyme by enzyme-bound substrate, is reported in this communication. The inactivation of β-cyclopiazonate oxidocyclase by diethylpyrocarbonate and its reactivation by hydroxylamine is demonstrated. The effect of the carbethoxylation of histidyl residues on the interaction between the enzyme and its substrate, β-cyclopiazonic acid, is discussed.

MATERIALS AND METHODS

Materials

β- and α-cyclopiazonic acid were obtained as described [29, 30]. Tryptophan tetramate (3-acetyl-5-scatyltetramic acid) was prepared as described in ref. 31. 4-γ,γ-dimethylallyl-tryptophan was prepared according to the method of Plieninger et al. [32]. Tryptamine was obtained from BDH Chemical Limited, Poole, England, L-tryptophan from Merck A.G., Darmstadt, Germany, and indole propionic acid and diethyl pyrocarbonate from K and K Laboratories, Plainview, N.Y., Hollywood. The

isolation, purification and assay of the β-cyclopiazonate oxidocyclase isoenzymes have been described [19, 21]. Either isoenzyme 1.3 or isoenzyme 3, which at the time of study had specific activities of 110 nmoles · min^{-1} · mg $enzyme^{-1}$ and 121 nmples · min^{-1} · mg $enzyme^{-1}$, when using 2,6-dichlorophenolindophenol as an electron acceptor, was used in this study. The spectral properties of these isoenzymes have been reported [21, 28]. The isoenzymes were stored in a 4:1 mixture of 0.05 M sodium phosphate, pH 6.8, and ethylene glycol. The ethylene glycol was routinely removed, prior to using the enzyme for experiments, by gel chromatography on Sephadex G-25.

Enzyme assay and spectrophotometric titration methods

The conditions of enzyme assay and the spectrophotometric titration of the enzyme with substrate analogues have been described [21].

The influence of pH on the *x*- and *y*-intercepts of Benesi–Hildebrand plots [21] of the spectrophotometric titration data for the binding of various substrate analogues to the enzyme was studied as follows: 0.5 ml of the enzyme in 0.05 M sodium phosphate buffer, pH 6.8, was added to 2 ml of a McIlvaine buffer of the desired pH obtained by mixing appropriate volumes of 0.1 M citric acid and 0.2 M disodium hydrogenphosphate. Above pH 8.0 an 0.2 M Na_2CO_3–$NaHCO_3$ buffer was used. The pH of the enzyme solution was measured accurately by means of a Radiometer pH meter immediately after the spectrophotometric titration of the enzyme with the substrate analogue.

Spectrophotometric determinations were performed on an Aminco Chance Dual Wavelength Splitbeam Recording Spectrophotometer and at room temperature i.e. 25 °C unless otherwise stated.

Determination of the spectrum of the enzyme substrate intermediate

Because of the low catalytic activity of the enzyme [21] the spectrum of the enzyme–substrate intermediate could be determined by conventional spectrophotometry. For this purpose it was essential to evaluate whether the reoxidation of the enzyme by atmospheric O_2 was significantly fast by comparison to its reduction by substrate. The spectrum of the reduced enzyme in 0.05 M phosphate buffer, pH 6.8, was determined after the addition of β-cyclopiazonic acid to the enzyme under aerobic conditions to give final concentrations of 60.6 μM β-cyclopiazonic acid and 7.25 μM enzyme. The experiment was repeated in a Thunberg cuvette. The gaseous phase above the enzyme solution was repeatedly evacuated and flushed with nitrogen prior to adding β-cyclopiazonic acid from the side arm. There was no significant difference between the spectra which were obtained under aerobic conditions and at much lower O_2 concentrations. The enzyme therefore appeared to recycle in the completely reduced form with O_2 as an electron acceptor.

The spectrum of the enzyme substrate intermediate was therefore determined under aerobic conditions at 14 °C. The enzyme reaction was started by the injection of 1.0 ml of enzyme from a thermostated syringe into 1.5 ml of a β-cyclopiazonic acid solution. Both solutions were 0.05 M with respect to sodium phosphate, pH 6.8, and the final concentrations of enzyme and β-cyclopiazonic acid were 14.4 μM and 0.303 mM respectively. Under these conditions the time interval required for the formation of the enzyme-substrate complex may be neglected by comparison to the much slower phase involving the reduction of the enzyme to the fully reduced form [21]. The latter process obeyed first order kinetics and was independent of β-cyclopiazonic acid

concentration. This allowed for the determination of the spectrum of the enzyme-substrate intermediate as described by Cattalini et al. [33].

The pH dependence of the reduction of the enzyme

The first order rate constant for the intramolecular reduction of the flavin coenzyme by enzyme-bound substrate was determined at various pH values by injection of the enzyme into a McIlvaine buffer of a suitable pH. The pH of the reaction mixture was determined once the reaction had reached completion.

The inactivation of the enzyme by diethylpyrocarbonate

The enzyme was contained in a 1:1 mixture of 0.05 M sodium phosphate buffer, pH 6.8, and a McIlvaine buffer of pH 6.0 which was prepared as described above. The resultant pH was 6.15. The carbethoxylation of histidyl residues was estimated by following the change in adsorbance at 242 nm when 10 μl of a methanolic solution of diethyl pyrocarbonate was reacted with 1.2 ml of the enzyme solution contained in one segment of a tandem cuvette, in the usual tandem cell arrangement [34, 35]. The concentration of the carbethoxylated histidyl residues was calculated by assuming a molar extinction coefficient of 3200 at 242 nm [36].

The inactivation of the enzyme by diethylpyrocarbonate was followed by performing enzyme assays at suitable intervals.

The concentration of diethylpyrocarbonate in methanolic stock solutions was estimated by determining the change in absorbance when 10 μl of the diethylpyrocarbonate solution was reacted with 2.5 ml of 25 mM histidine monohydrochloride in a McIlvaine buffer of pH 6.0.

The reactivation of the carbethoxylated enzyme by hydroxylamine

The enzyme was treated with diethylpyrocarbonate until a 90% inhibition of activity, and the solution was then made 0.5 M with respect to hydroxylamine by the addition of an appropriate volume of a 2-M hydroxylamine solution, the pH of which was previously adjusted to 7.0 with NaOH. At different times hydroxylamine, which interferes with the enzyme assays using 2,6-dichlorophenolindophenol as an electron acceptor, was removed by gel chromatography and the overall percentage of recovery of the original enzyme activity in the effluent fractions from a Sephadex G-25 column was estimated.

The effect of β- and α-cyclopiazonic acid on the visible spectrum of the carbethoxylated enzyme

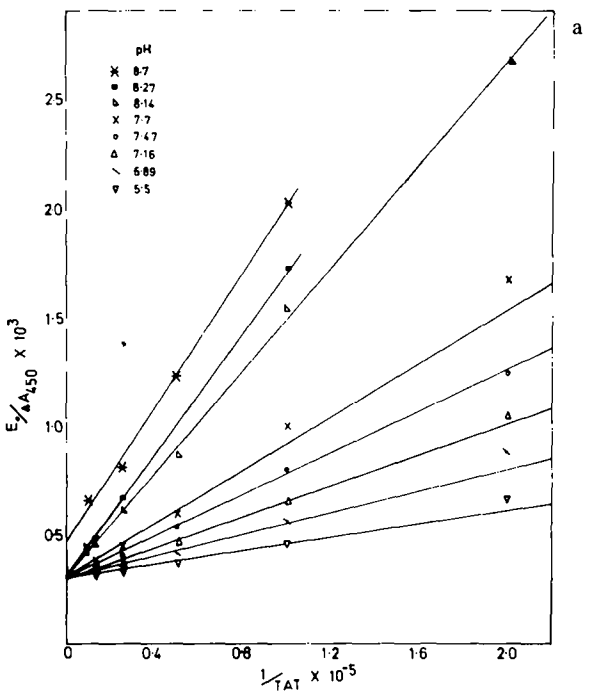
The enzyme was reacted with diethylpyrocarbonate to obtain more than 90% inhibition of activity. Unreacted diethylpyrocarbonate was then removed by gel chromatography on Sephadex G-25 and the effect of β- and α-cyclopiazonic acid on the spectrum of the oxidized enzyme was examined by spectrophotometric titration.

RESULTS AND DISCUSSION

The pH dependence of the Benesi–Hildebrand plots for the binding of substrate analogues to β-cyclopiazonate oxidocyclase

Benesi–Hildebrand plots for the binding of tryptophan tetramate, L-tryptophan, tryptamine, tryptophan methyl ester and indole propionic acid at various pH values

are shown in Fig. 1. For the sake of clarity only a limited number of curves are presented. In the case of tryptophan tetramate there appears to be a slight positive deviation from linearity at the lowest concentrations of tryptophan tetramate and at low pH



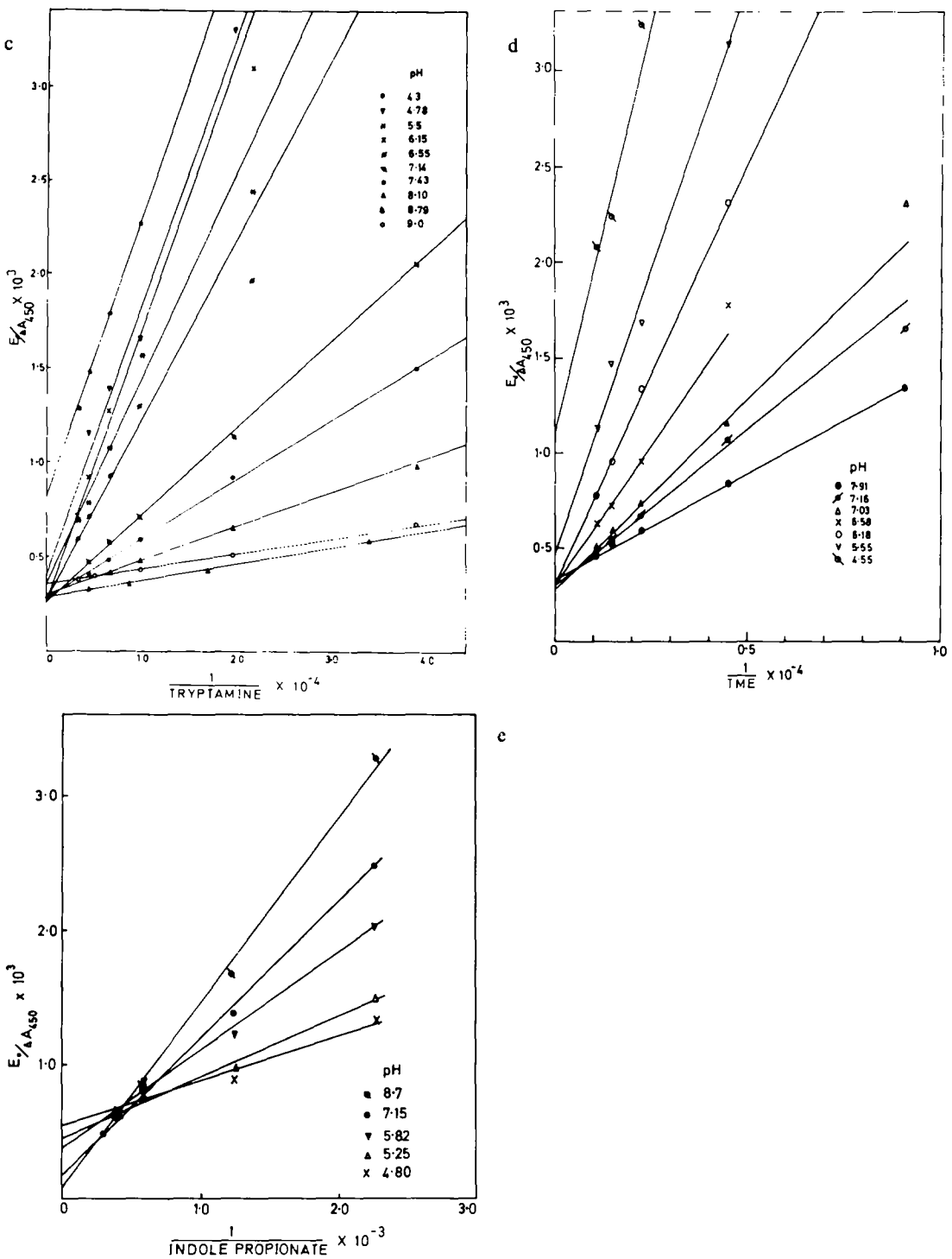


Fig. 1. Benesi–Hildebrand plots of the aerobic spectrophotometric titration of β-cyclopiazonate oxidocyclase (isoenzyme 1.3) with substrate analogues: (a) tryptophan tetramate (TAT), (b) L-tryptophan, (c) tryptamine, (d) tryptophan methyl ester (TME), (e) indole propionic acid. The pH values are indicated. Some of the data points at high $E/\Delta A_{450\ nm}$ values are for ease of representation not shown in Fig. 1, but were used in simple linear regression.

values, in agreement with a modified Benesi–Hildebrand equation [21]. Whereas the slopes of the curves for tryptophan tetramate, L-tryptophan and indole propionic acid increase with increasing pH, an opposite effect is observed with the positively charged indole derivatives, tryptamine and tryptophan methyl ester.

Curves of log K_D, as obtained from the x-axis intercepts of the Benesi–Hildebrand curves of Fig. 1, vs pH are shown in Fig. 2. Because of the inaccuracy due to the variation of the y-axis intercepts of Benesi–Hildebrand plots for the binding of indole

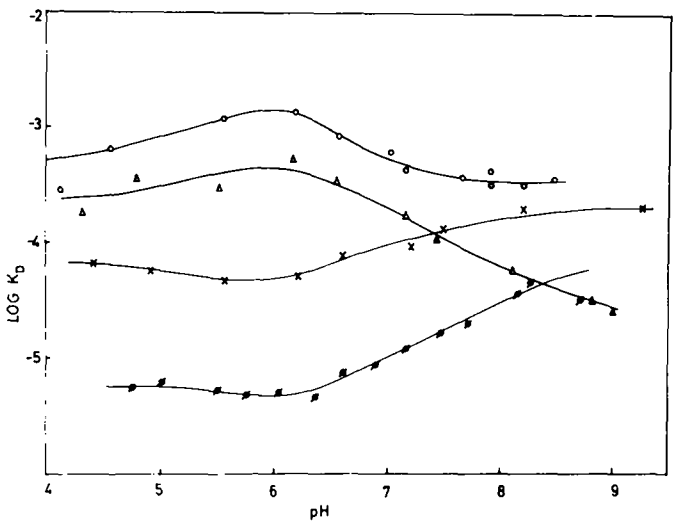


Fig. 2. The logarithm of the dissociation constant, K_D, of the complexes of L-tryptophan (×—×), tryptophan tetramate (○—○), tryptamine (∅—∅) and tryptophan methyl ester (△—△) as a function of pH.

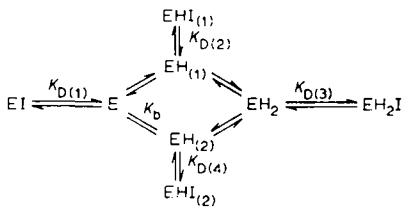
propionic acid to the enzyme, the log K_D vs pH curve for this substrate analogue is not shown in Fig. 2. Whereas the overall pH dependence for the binding of indole propionic acid is similar to that of tryptophan tetramate and L-tryptophan, the lack of one amino group, however, has the effect of increasing the dissociation constant by approximately an order of magnitude relative to L-tryptophan. This finding is particularly difficult to reconcile with any attempt to explain the data of Fig. 2 in terms of a single ionizing group on the enzyme, and the slight bell-shaped form of the curves is also not in agreement with such a simple model.

Since it may be inferred from the effects of these indole derivatives on the visible spectrum of the enzyme-bound flavin that these compounds all bind at the active site of the enzyme, they must to a greater or lesser extent undergo interactions with the same groups on the enzyme. Whereas it is possible to apply the theory of Dixon and Webb [37] to each of the curves of Fig. 2 individually, this theory leads to conflicting pK'_a values for a group on the free enzyme or on the enzyme–substrate analogue complex when one compares the values obtained for the binding of different substrate analogues.

Moreover, the underlying assumption that binding of the substrate analogue would be restricted to one particular ionization state of the enzyme implies a prepon-

derance of electrostatic charge effects in binding phenomena which can hardly be expected to exist [38] especially if there are indications that the binding site is essentially hydrophobic in nature [21].

It therefore seems necessary to propose as a minimal hypothesis, the presence of two ionizing groups in the active site of the enzyme. For such a system one could write a scheme showing the interaction between indole derivatives, I, and the enzyme, E, in different states of protonation.



Scheme 1.

For this scheme one can easily derive a pH-dependent form of the Benesi–Hildebrand equation:

$$\frac{E_0}{\Delta A} = k\left\{1 + \left(\frac{1 + \frac{K_b}{H^+} + \frac{K_b}{K_a} + \frac{H^+}{K_c}}{\frac{K_b}{H^+ K_{D(4)}} + \frac{1}{K_{D(2)}} + \frac{K_b}{K_a K_{D(4)}} + \frac{H^+}{K_c K_{D(3)}}}\right)\frac{1}{I}\right\} \qquad (1)$$

$k = 1/(\varepsilon_E - \varepsilon_{EM})$, where ε_E and ε_{EM} are the molar extinction coefficients of the free enzyme and the enzyme–indole derivative complex respectively.

Theoretical curves of log K_D vs pH are shown in Fig. 3 for arbitrary values of the proton dissociation constants K_a, K_b and K_c and the dissociation constants of the substrate analogue complexes, $K_{D(i)}$. A closer agreement between the theoretical curves in Fig. 3 and the experimental curves of Fig. 2 would necessitate a parameter search which we have not attempted. It is apparent, though, that the pH dependence of the dissociation constants in Fig. 2 could be attributed to the presence of at least two ionizing groups with pK_a values in the region 6.0 to 7.0 in the active site of the enzyme. These findings may therefore imply indirectly the presence of two histidyl residues in the active site of β-cyclopiazonate oxidocyclase. At low pH values positively charged indole derivatives may then be expected to bind weakly to the enzyme whereas at alkaline pH values interactions between the negatively charged "pyridine-like" nitrogen of the imidazole ring and the positive amino group [39, 40] would result in an increased affinity of indole derivatives such as tryptophan methyl ester and tryptamine for the enzyme. The observed pH dependence for the binding of tryptophan tetramate is similarly consistent with the presence of imidazole groups in the active site. However, since carboxyl groups with pK_a values as high as 7.2 have been reported [41], the data is also not inconsistent with the presence of one histidyl and one carboxyl group in the active site of the enzyme.

The major advantage of analysing the pH dependence of the binding of a number of substrate analogues to an enzyme seems to be that one can on the basis of

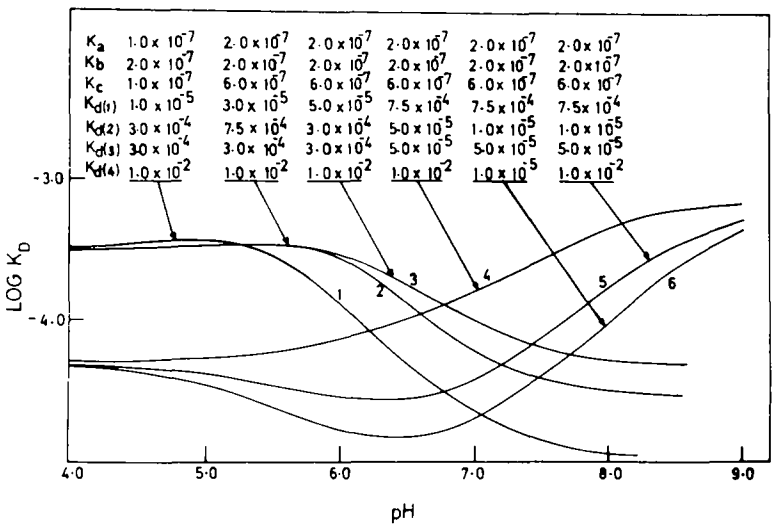


Fig. 3. Theoretical curves for arbitrary values of the constants in Scheme I of the logarithm of the overall dissociation constant, K_D, as calculated from Eqn 1 as a function of pH.

such an analysis discard simple models, which might have explained the data for a single substrate, as inadequate. It is obvious however that even a study of the binding of several substrate analogues to an enzyme does not yield data which can readily be interpreted in an unambiguous manner.

It may also be of interest to draw attention to a further complication which arises in this type of analysis due to the variation of the *y*-axis intercepts of the Benesi–Hildebrand curves. Except at the highest pH value, the *y*-axis intercept for the binding of tryptophan tetramate and L-tryptophan is independent of pH. The increased *y*-axis intercepts at the highest pH value was also observed in the case of tryptamine and could possibly be attributed to the ionization of the N-3 proton of the isoalloxazine ring. The curves for tryptamine and particularly for tryptophan methyl ester, however, also exhibit increased *y*-axis intercepts at low pH values. This variation in the *y*-axis intercepts may indicate a transition between two or more types of complexes of the substrate analogue with the enzyme [42, 43]. This interpretation was substantiated by spectrophotometric titration data. At neutral pH values the spectrophotometric titration of the enzyme with tryptophan methyl ester followed an isosbestic course, but at pH 4.8, the titration was non-isosbestic, thus indicating that more than one complex of the enzyme with this substrate analogue is formed at low pH values. Such a phenomenon would result in an overestimation of the affinity of the enzyme for the substrate analogue [42]. An analogous situation would exist in steady state kinetic determinations of K_D values with the important difference that one would not be able to tell whether decreased K_D values are due to the formation of several different complexes of an enzyme with a ligand [43].

In the case of indole propionic acid an opposite effect is observed: weaker binding of the indole derivative results in a pronounced lowering of the *y*-axis intercept, thus rendering the determination of dissociation constants very inaccurate. This phenomenon may be explained on theoretical grounds: As the binding of a ligand to an

an enzyme becomes progressively weaker, the ligand will gradually begin to act as a perturbant rather than as a complexing agent. When the effect of the perturbant is to cause a hypo- or hyperchromicity of an absorption band which is linearly related to the perturbant concentration, plots of the data according to the method of Benesi and Hildebrand will pass through the origin to give the impression of an infinite absorbance difference at infinite perturbant concentration. The situation is to some extent analogous to that of contact charge transfer [42]. The interaction of indole propionic acid with the flavoquinone enzyme resulted in a general hypochromicity of the visible absorbance bands of the flavin and no isosbestic points, which might have indicated complex formation as opposed to a mere perturbation of the flavin chromophore, were observed. The pH dependence of the y-axis intercepts of Benesi–Hildebrand plots for the binding of indole propionic acid to the enzyme is therefore possibly due to a variation in the extent to which this indole derivative functions either as a perturbant or as a complexing agent at different pH values.

The pH and temperature dependence of the first order rate constant for the reduction of the enzyme

The reduction of the flavoenzyme by enzyme-bound substrate exhibited virtually no pH dependence [Fig. 4] and the slight wave in the curve could perhaps reflect the ionization of the N-1 proton of the flavohydroquinone [44]. The limited pH

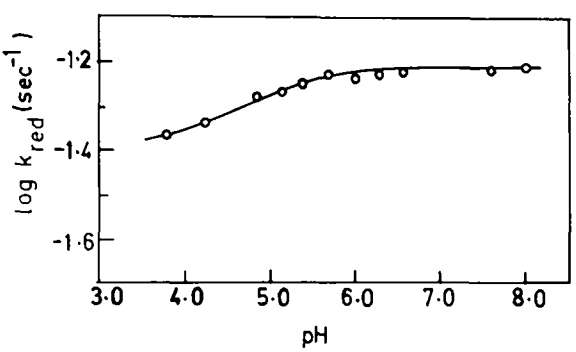


Fig. 4. Dependence of the first order rate constant, k_{red}, for the reduction of isoenzyme 1.3 by enzyme bound β-cyclopiazonic acid on pH. The reaction was initiated by the injection of 1.0 ml enzyme solution into 1.5 ml β-cyclopiazonic acid solution to give final concentrations of 14.4 μM enzyme and 0.304 mM β-cyclopiazonic acid. Because of the limited solubility of β-cyclopiazonic acid, a concentration of 0.152 mM β-cyclopiazonic acid was used below pH 4.3. The temperature was 14.3 °C and the reduction was followed by observing the decrease in absorbance at 450 nm.

dependence in the reduction step is similar to that found by Bright and Appleby in the reduction of glucose oxidase by enzyme-bound 2-deoxyglucose [45] and support the interpretation that the bends in curves of log K_D vs pH (Fig. 2) for the substrate analogues most probably does not reflect ionizations of the enzyme-substrate analogue complexes.

At 30 °C the first order rate constant for reduction has a value of 0.126 s^{-1} giving a maximum turnover of 7.55 moles substrate per mole enzyme per min. This value corresponds fairly well with the catalytic activity of approximately 6.0 moles

substrate per mole enzyme per min when 2,6-dichlorophenolindophenol is used as an electron acceptor. The reduction of the flavoenzyme by enzyme-bound substrate therefore seems to be the rate limiting step in catalysis with 2,6-dichlorophenolindophenol as electron acceptor. This is further born out by the relatively high activation energy of 11 400 cal/mole as calculated from Fig. 5

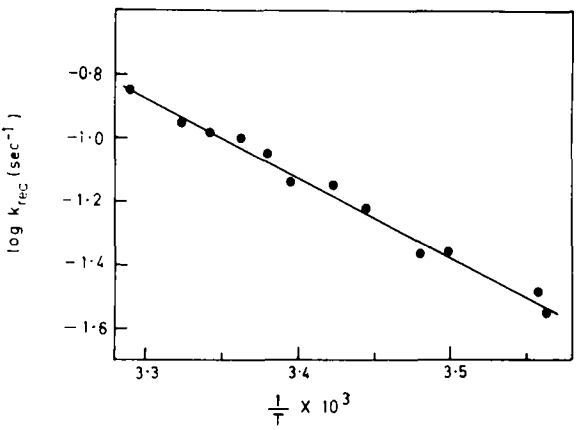


Fig. 5. Dependence of the first order rate constant for the reduction of isoenzyme 1.3 by enzyme bound substrate on temperature at pH 6.8 in 0.05 M sodium phosphate. For experimental conditions see legend to Fig. 4 and text.

The inactivation of β-cyclopiazonate oxidocyclase by diethylpyrocarbonate

The enzyme was rapidly inactivated by relatively low concentrations of diethylpyrocarbonate (Fig. 6). Partial protection, even at higher concentrations of diethylpyrocarbonate, was afforded by α-cyclopiazonate. The inactivation of the enzyme was

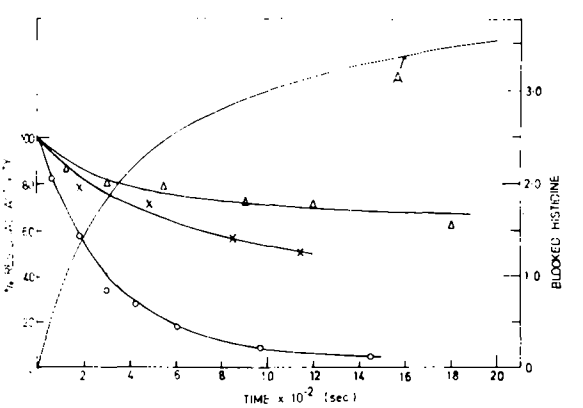


Fig. 6. Inactivation of β-cyclopiazonate oxidocyclase by diethylpyrocarbonate at pH 6.15 and 10 °C. The inactivation was effected by reacting (i) 19.7 μM isoenzyme 3 with 0.195 mM diethylpyrocarbonate (○—○) (ii) 19.7 μM isoenzyme 3 with 0.533 mM diethylpyrocarbonate in the presence of 0.1425 mM α-cyclopiazonic acid (×—×) and (iii) 10 μM enzyme with 0.288 mM diethylpyrocarbonate in the presence of 95 μM α-cyclopiazonic acid. (△—△) Curve A represents the progress of carbethoxylation of β-cyclopiazonate oxidocyclase under the same conditions as for (i) as estimated from the increase in absorbance at 242 nm.

accompanied by an increase in absorbance at 242 nm (Curve A). A complete loss of activity was obtained by the modification of approximately three histidyl residues per mole of enzyme-bound flavin (Fig. 7).

Diethylpyrocarbonate treatment had no effect on the visible spectrum of the enzyme. Thome-Beau et al [14] also reported that diethylpyrocarbonate does not appear to react with FAD.

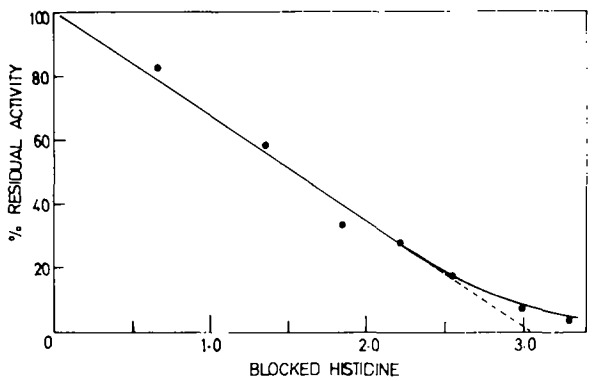


Fig. 7. The percentage residual activity of β-cyclopiazonate oxidocyclase as a function of the number of blocked histidyl residues.

The effect of carbethoxylation on the binding of β-cyclopiazonic acid to the enzyme

Previous work [28] had shown that there appears to be very little difference between the K_D values for the binding of substrate analogues to β-cyclopiazonate oxidocyclase in which the flavin had been reduced by borohydride and those for the native flavoquinone enzyme. On the other hand modification of the apoenzyme protein by carbethoxylation results in a marked decrease in affinity for indole derivatives.

The effect of β-cyclopiazonic acid on the visible spectrum of the carbethoxylated enzyme is shown in Fig. 8. A dissociation constant of 0.161 mM was calculated from the Benesi–Hildebrand plot which is shown in Fig. 9. This value may be compared with the dissociation constant of the complex of the borohydride-reduced enzyme with β-cyclopiazonic acid which was estimated at 3.3 μM. Carbethoxylation of the enzyme has therefore resulted in a considerable increase in the dissociation constant of the enzyme–substrate complex. We have also tried to determine the K_D value for the binding of α-cyclopiazonic acid to the carbethoxylated enzyme, but the binding was so weak that the limited solubility of α-cyclopiazonic acid did not allow us to obtain an accurate value.

The spectra of the flavoquinone and flavohydroquinone forms of the enzyme as compared to the spectrum of the enzyme–substrate intermediate complex formed by the native enzyme is shown in Fig. 10. The latter spectrum bears a striking resemblance to the spectra of the complex between the enzyme and α-cyclopiazonic acid [21] or 4-γ,γ-dimethylallyltryptophan (Fig. 11).

Comparison of the spectrum of the enzyme–substrate complex during catalysis by the native enzyme (Fig. 10) and the spectral changes observed during spectrophotometric titration of the carbethoxylated enzyme with substrate (Fig. 8) indicates that

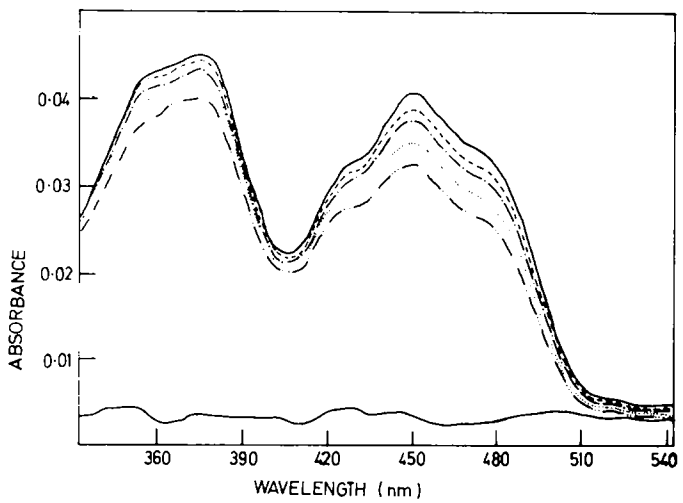


Fig. 8. Aerobic spectrophotometric titration of carbethoxylated β-cyclopiazonate oxidocyclase with β-cyclopiazonic acid. The curves represent the spectra of oxidized isoenzyme 3 (solid line) and in the presence of 18.9 μM β-cyclopiazonic acid (— — —), 37.8 μM β-cyclopiazonic acid (·–·–·), 75.7 μM β-cyclopiazonic acid (·····) and 151.5 μM β-cyclopiazonic acid (–···–···–) all in a 0.05-M sodium phosphate buffer, pH 6.8.

carbethoxylation of the enzyme has altered the properties of the complex which it can form with β-cyclopiazonic acid; apparently in such a way that the flavoenzyme can no longer be reduced by the enzyme-bound substrate.

Reactivation of the carbethoxylated enzyme with hydroxylamine

The enzyme was progressively reactivated over a period of 4 h upon incubation with hydroxylamine at room temperature (Table I).

During these studies it was also noticed that the flavocoenzyme is visibly bleached in the presence of hydroxylamine. Further investigation showed that the

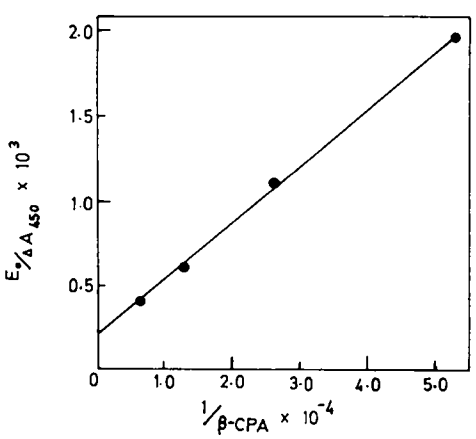


Fig. 9. Benesi–Hildebrand plot of the spectrophotometric titration of carbethoxylated β-cyclopiazonate oxidocyclase with β-cyclopiazonic acid.

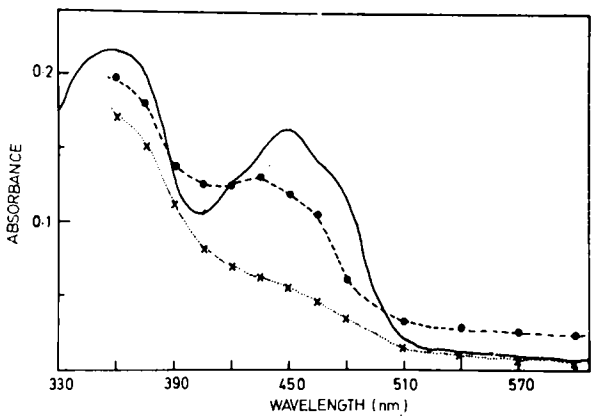


Fig. 10. Comparison of the visible spectra of the oxidized isoenzyme 1.3 (solid curve), the enzyme–β-cyclopiazonic acid–intermediate complex (○---○) and the enzyme after reduction with excess substrate (× · ×). For method of determination see Materials and Methods.

bleaching of the flavocoenzyme is accompanied by the appearance of a spectrum resembling that of an anionic semiquinone.

The formation of the neutral semiquinone form of glucose oxidase by treatment with hydroxylamine has been reported by Swoboda and Massey [46].

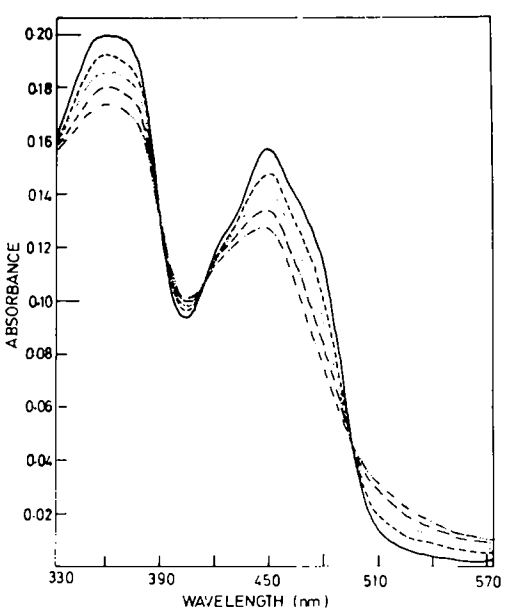


Fig. 11 Aerobic spectrophometric titration of β-cyclopiazonate oxidocyclase with 4-γ,γ-dimethylallyltryptophan. The curves represent the spectra of the oxidized isoenzyme 1.3 as such (solid line) and in the presence of 15.2 μM (— — —), 30.4 μM (· · · · · ·), 60.8 μM (· – · – ·) and 121.6 μM 4-γ,γ-dimethylallyltryptophan (· · – · · – · ·), all in 0.05 M sodium phosphate buffer, pH 6.8, and at 21 °C.

TABLE I

REACTIVATION OF THE CARBETHOXYLATED ENZYME WITH HYDROXYLAMINE

The enzyme at a concentration of 15.7 μM enzyme-bound FAD was reacted with 0.222 mM diethylpyrocarbonate in a volume of 3 ml for 20 min to obtain approximately 90% inhibition of activity. Reversion of inhibition was obtained by incubation of the enzyme for various periods of time with hydroxylamine. (See Materials and Methods)

Time (h)	Activity recovered (%)
0.5	32
3.0	71
4.0	94

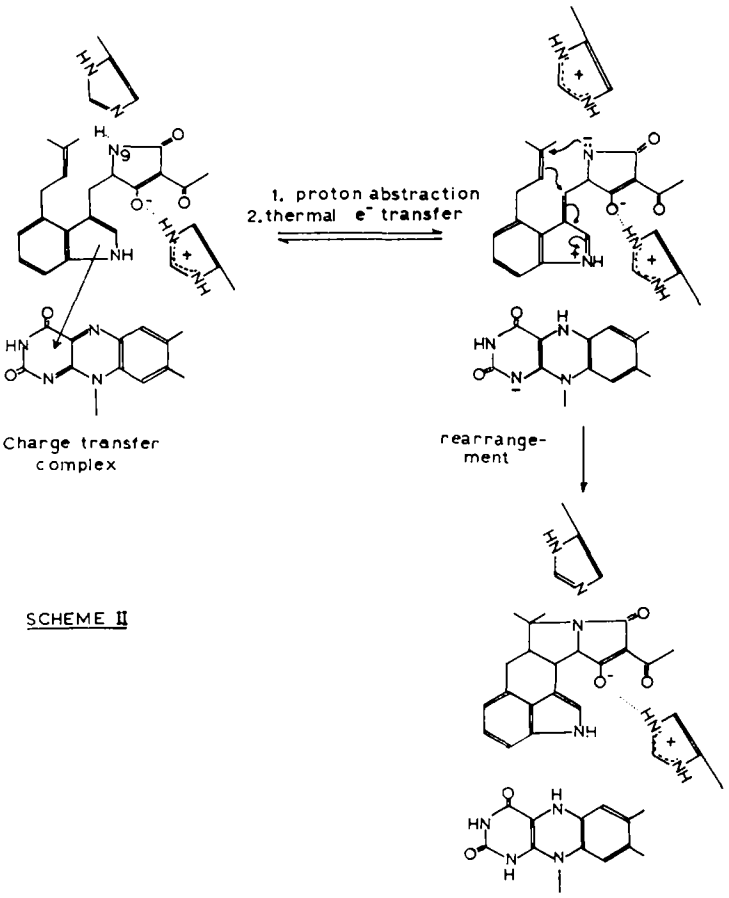
CONCLUSIONS

It was proposed [20] that the oxidocyclization reaction involves a nucleophilic attack by N-9 of the tetramic acid ring on a carbonium ion reaction intermediate of the dimethylallyl group. Since, however, N-9 is essentially an amide nitrogen it cannot be regarded as a strong nucleophile. The results which were obtained by studying the pH dependence of the binding of indole derivatives to β-cyclopiazonate oxidocyclase and the chemical modification studies using diethylpyrocarbonate indicate that histidyl residues may not only play an important role in the binding of the substrate, but also in the activation of the substrate during the formation of the enzyme–substrate intermediate complex. If one considers the possibility that the substrate is activated by withdrawal of a proton from N-9 of β-cyclopiazonic acid, then the enhanced nucleophilic character of N-9 of the tetramic acid ring will allow it to act as an electron source towards the dimethylallyl group in a concerted ring closure reaction as depicted in Scheme II. The reaction could then be initiated by electron donation from the indole ring towards the flavin [47, 48], followed by proton abstraction from N-9 and a nucleophilic attack of N-9 on the dimethylallyl group.

The hypothesis that a histidyl residue functions to activate the substrate by proton removal is, therefore, attractive. The finding that 4-γ,γ-dimethylallyltryptophan forms a complex with the enzyme, but fails to reduce the flavin could reflect the unsuitability of a positively charged amino group for appropriate activation by proton abstraction. In view of the high activation energy for reduction of the enzyme by enzyme-bound substrate it is possible that any failure to "activate" the substrate during binding will make this step impossible.

The first order rate constant for the reduction of the enzyme by enzyme-bound substrate, however, shows only a very limited pH dependence, contrary to what one would expect if the reduction is initiated by proton abstraction by a histidyl residue. Consequently such an activation of the substrate by proton removal is more likely to occur during the formation of the enzyme-substrate complex.

The difference between the spectra of the enzyme–substrate complexes formed by the native and carbethoxylated enzymes, respectively, indicates a marked effect of carbethoxylation of histidyl residues on the nature of the interaction of β-cyclopiazonic acid with the enzyme. This is of considerable interest and could perhaps



SCHEME II

indicate a very close co-operativity between histidyl residues and the flavocoenzyme to form the "correctly activated" enzyme–substrate complex.

It was pointed out by Huc et al. [15] that diethylpyrocarbonate cannot be regarded as sufficiently specific to distinguish between essential and non-essential histidyl residues in the enzymes octopine dehydrogenase and lactate dehydrogenase. In the case of lactate dehydrogenase the difficulty was resolved by Holbrook and Ingram [49] by relying on the rapidity of the reaction between the essential histidyl residue of lactate dehydrogenase and diethylpyrocarbonate. Unfortunately the carbethoxylation of the histidyl residues of β-cyclopiazonate oxidocyclase is not sufficiently fast to disregard the spontaneous decomposition of diethylpyrocarbonate in water, and the inactivation studies can therefore only provide qualitative evidence for the presence of histidyl residues in the active centre.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Council for Scientific and Industrial Research in Pretoria for financial assistance, to the Department of Chemical

Pathology, Medical School, University of Natal for the use of equipment during the initial stages of some of this work, and to the Department of Biometry, Faculty of Agriculture, University of Natal for the use of simple linear regression program.

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