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SUBSTRATE SPECIFICITY OF CAERULOPLASMIN INDOLES AND INDOLE ISOSTERES

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Abstract—Several indoles and indole isosteres have been examined as potential substrates for the oxidative enzyme caeruloplasmin. Amongst the indoles the presence of a hydroxy group on the aromatic ring of the indole nucleus is a necessary requirement for substrate activity; this hydroxy group may be at position 4, 5 or 6 of the indole nucleus. An aminoalkyl, or substituted aminoalkyl, chain attached to position 3 of the indole ring is necessary for high substrate activity; the distance separating the basic group of the side chain from the indole ring does not appear to be critical. Studies on indole isosteres (analogues of 5-hydroxytryptamine) have clearly shown that only the presence of an imino group, at position 1 of the bicyclic system, is compatible with substrate activity.

IN PREVIOUS papers[1,2] it was shown that certain centrally active drugs interacted with the oxidative enzyme caeruloplasmin in such a way as to indicate that this enzyme, or one with similar properties, might play an important role in the normal functioning of the central nervous system. These results led to the conclusion that more detailed investigations on caeruloplasmin were desirable, particularly kinetic studies leading to a clarification of its mechanism of action. Work with similar aims on other enzymes, especially esterases such as chymotrypsin,[3] has repeatedly demonstrated the importance of first defining their substrate specificity as a necessary preliminary to detailed kinetic and mechanistic studies, and it was thought that caeruloplasmin would be no exception. Although several papers dealing with this aspect of work on caeruloplasmin have appeared[4–8] it was felt that the subject had not been sufficiently explored, particularly with regard to substrates related to the biogenic amines dopamine, adrenaline, noradrenaline and 5-hydroxytryptamine. Such substrate specificity studies on caeruloplasmin are perhaps especially important in view of the role which we have suggested for an enzyme such as caeruloplasmin in the normal functioning of the central nervous system.[1,2] The existing information on the substrate specificity of caeruloplasmin has therefore been extended with particular reference to analogues of catecholamines and 5-hydroxytryptamine. A previous paper[9] has dealt with the substrates analogous to catecholamines; the present paper deals with substrates related to 5-hydroxytryptamine.

METHODS

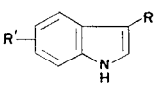
Materials. Caeruloplasmin from human plasma was purchased from A. G. Kabi Ltd. (Sweden) as a 5 per cent aqueous solution. The compounds used were either obtained from commercial sources or, where not commercially available, were synthesised in our laboratories following published procedures. In the latter cases the

identities of the compounds were confirmed by melting points, elemental analyses and spectroscopic methods (infrared and nuclear magnetic resonance spectroscopy).

Enzyme studies. The rate of oxygen uptake during the enzyme-catalysed reactions was measured polarographically using a modified Clark electrode[10] in conjunction with a Radiometer Polarograph Type PO4. The oxidation of reduced nicotineamide adenine dinucleotide (NADH) to oxidized nicotineamide adenine dinucleotide (NAD^+) by caeruloplasmin in the presence of these compounds was measured by following the rate of formation of NAD^+ polarographically using a Differential Cathode Ray Polarograph Type A1660 (Southern Analytical Instruments) and also by following the disappearance of NADH spectrophotometrically. Detailed experimental procedures have been described previously.[1]

RESULTS

The results of these investigations are summarized in Tables 1 and 2. The K_m values quoted in the Tables are based on the rate of oxidation of NADH to NAD^+ and not on the rate of oxygen uptake. In certain cases, noted in Table 1, the assay solution rapidly became turbid as the enzyme catalysed reaction proceeded, thus effectively preventing the determination of K_m values even though the compounds were obviously substrates.

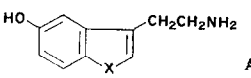
TABLE 1. INDOLE SUBSTRATES  FOR CAERULOPLASMIN

Compound no.	R	R′	Substrate action: O_2 Uptake	Substrate action: NADH Oxidation	Remarks
1	$H_2N(CH_2)_2$	H	—	—	
2	$CH_3NH(CH_2)_2$	H	—	—	
3	$H_2N(CH_2)_2$	4—OH	+	+	Solution clouds
4	$H_2N(CH_2)_2$	4—OCH_3	—	—	
5	H	5—OH	—	—	
6	$H_2N(CH_2)_2$	5—OH	+	+	$K_m = 4 \times 10^{-4}$M
7	$H_2N(CH_2)_3$	5—OH	+	+	$K_m = 3 \times 10^{-4}$M
8	$H_2N(CH_2)_4$	5—OH	+	+	$K_m = 5 \times 10^{-4}$M
9	$CH_3NH(CH_2)_2$	5—OH	+	+	$K_m = 3{\cdot}5 \times 10^{-4}$M
10	$(CH_3)_2N(CH_2)_2$	5—OH	+	+	$K_m = 5 \times 10^{-4}$M
11	$H_2N(CH_2)_2$	5—OCH_3	—	—	
12	$H_2N(CH_2)_2$	6—OH	+	+	Solution clouds
13	$H_2N(CH_2)_2$	6—OCH_3	+	+	O_2 uptake in presence of NADH*
14	$H_2N(CH_2)_2$	7—OCH_3	—	—	
15	$H_2N(CH_2)_2$	5,6-$(OH)_2$	+	+	Solution clouds
16	$H_2N(CH_2)_2$	5,6-$(OCH_3)_2$	+	+	*

* Insufficient material available to measure K_m.

DISCUSSION

The structural variations which have been made amongst the substrates described in this paper comprise substitution of hydroxy or methoxy groups into the benzene ring of the indole nucleus, modifications to the tryptamine side chain and replacement of the indole imino group by other heteroatoms. These will be discussed in turn.

TABLE 2. 5-HYDROXYTRYPTAMINE ISOSTERES  AS SUBSTRATES FOR CAERULOPLASMIN

Compound no.	X	Substrate action: O_2 Uptake	Substrate action: NADH Oxidation	Remarks
6	NH	+	+	$K_m = 4 \times 10^{-4}$M
17	CH_2	−	−	Inhibits noradrenaline and 5-HT oxidation, $K_i = 5 \times 10^{-5}$M
18	O	Very slow	Very slow	No effect on noradrenaline oxidation. Inhibits 5-HT oxidation, $K_i = 3 \times 10^{-3}$M
19	S	Very slow	Very slow	No effect on the oxidation of noradrenaline or 5-HT

The results given in Table 1 show that a hydroxy group in the benzene ring of the indole nucleus of tryptamines is a necessary prerequisite for high substrate activity, tryptamine itself (compound 1) and its 4-, 5- and 7-methoxy derivatives (compounds 4, 11 and 14 respectively) possessing no substrate activity whilst the 4- and 5-hydroxytryptamines (compounds 3 and 6 respectively) were very good substrates for this enzyme. Two exceptions to this statement were, however, noted, the 6-methoxy and 5,6-dimethoxytryptamines (compounds 13 and 16) apparently being substrates for this enzyme. No explanation for this anomaly can be offered at present, but it may be noted that both compounds were coloured solids which darkened rapidly on storage, even in the solid state, indicative of a high sensitivity to aerial oxidation in addition to enzymic oxidation.

Although the presence of a hydroxy group in the benzene ring of the indole nucleus is essential for high substrate activity the position of this hydroxy group appears to be relatively unimportant as witnessed by the fact that the 4-, 5- and 6-hydroxytryptamines (compounds 3, 6 and 12 respectively) are all substrates for this enzyme. It was unfortunate that in the case of compounds 3 and 12 the assay solutions rapidly became turbid thus precluding the measurement of K_m values; it is not possible, therefore, to make a direct quantitative comparison of compounds 3, 6 and 12 as substrates for this enzyme.

Comparison of 5-hydroxyindole (compound 5) with the 3-(ω-aminoalkyl) derivatives (compounds 6–10, Table 1) shows that a basic side chain at position 3 of the indole nucleus is essential for high substrate activity within the class of indole derivatives. Substitution on the side chain nitrogen atom, at least by methyl groups, appears to have relatively little effect on substrate activity (compare compounds 6, 9 and 10, Table 1). Although the presence of a basic side chain attached to position 3 of the indole nucleus is essential for high substrate activity, comparison of compounds 6, 7 and 8 (Table 1) shows that variations in the distance separating the basic group from the indole nucleus have very little effect on substrate activity. A similar conclusion was reached with regard to phenylalkylamine substrates for caeruloplasmin[9] and to this

extent there is a close resemblance between phenylalkylamine and indolealkylamine substrates for this enzyme.

In Table 2 are shown the results obtained from studies on 5-hydroxytryptamine isosteres as substrates for caeruloplasmin, and it is immediately apparent that only the presence of a nuclear imino group is compatible with substrate activity. The benzofuran (compound 18) and benzothiophene (compound 19) isosteres were such poor substrates that K_m values were not measured. Neither compound inhibited the oxidation of noradrenaline but the benzofuran did inhibit the oxidation of 5-hydroxytryptamine. Although this inhibition was rather weak ($K_i = 3 \times 10^{-3}$M) the fact that this compound did not inhibit the oxidation of noradrenaline at all may make it useful in studies on the mechanism of action of caeruloplasmin especially if, as seems possible, the oxidation of phenylalkylamines and hydroxytryptamines are mediated by different sites on the enzyme (see below).

A comparison of these results with those obtained previously on phenylalkylamine substrates[9] is instructive, the general points of resemblance between phenylalkylamine and tryptamine as substrates being particularly striking. For example both classes of substrates require the presence of hydroxy substituents in the aromatic ring for high substrate activity together with a basic side chain attached to the aromatic ring. In both cases variations of the distance separating the basic group of the side chain from the aromatic ring has relatively little effect on substrate activity and a similar comment may apply to changes in the position of substituent hydroxy groups in the aromatic ring, although this is less definitely established. These general similarities between substrates of the phenylalkylamine and tryptamine classes would indicate that they interact with a common site on the enzyme. However, whilst only one hydroxy group is necessary for high substrate activity with the tryptamines this is not the case with the phenylalkylamines, where a second oxygen function, either a hydroxy or methoxy group, is required. In addition, it has been shown that certain compounds, such as LSD[1] and some phenylalkylamines and amphetamines,[9] will differentially affect the caeruloplasmin-catalysed oxidation of noradrenaline and 5-hydroxytryptamine. The present work has shown that the benzofuran (compound 18, Table 2) will inhibit the oxidation of 5-hydroxytryptamine although it has no effect on the oxidation of noradrenaline. These observations strongly suggest that there are two sites on the enzyme, responsible for the oxidation of phenylalkylamines and tryptamines respectively; whilst these two sites have much in common they are clearly not identical. The work of Curzon and Speyer[11] on the effects of inorganic ions on the oxidation of *N,N*-dimethyl-*p*-phenylenediamine by caeruloplasmin also supports this suggestion. It would appear, therefore, that whilst there is evidence that the oxidation of phenylalkylamines and tryptamines is mediated by the same active site on the enzyme other results suggest that two sites are involved;[12,13] this dilemma will only be resolved by a more detailed study of the mechanism of action of caeruloplasmin, probably involving the use of some of the compounds which have been shown in this and the previous paper[9] to modify the enzymic oxidation of phenylalkylamines and tryptamines

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