Purification of Riboflavin Synthase by Affinity Chromatography using 7-Oxolumazines

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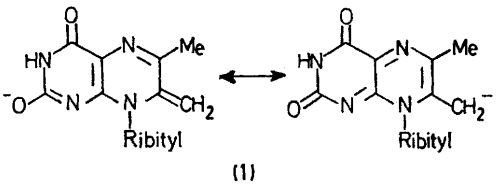
Summary Riboflavin synthase (EC. 2.5.1.9) from baker's yeast has been purified over 1000 fold by affinity chromatography using the putative transition-state analogue 6-carboxyethyl-7-oxo-8-D-ribityllumazine as the ligand attached to Sepharose 4B.

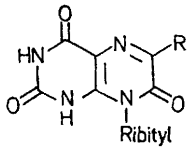
THE full potential of affinity chromatography as a tool for enzyme purification will only be realized when the necessary competitive enzyme inhibitors can be designed in a rational manner by consideration of the mechanism of the enzyme-catalysed reaction. We now report an example of the complete sequence of (a) elucidation of the mechanism of an enzyme-catalysed reaction, (b) application of this mechanism in the design of specific competitive enzyme inhibitors, and (c) utilization of these inhibitors for purification of the enzyme by affinity chromatography.

The mechanistic scheme studied[1] requires that one of the two molecules of 6,7-dimethyl-8-D-ribityllumazine involved in the formation of riboflavin be converted into a charge-delocalized anion (**1**) which then undergoes a series of further modifications to give riboflavin. According to transition-state analogue hypothesis,[2] compounds (**2**) similar in structure to the anion (**1**), should also bind strongly to riboflavin synthase.

In order to test this hypothesis several oxolumazines (**2**) have been synthesised by condensation of 5-amino-4-D-ribitylaminouracil with α-keto esters, and their inhibition characteristics have been determined using an extract of riboflavin synthase from baker's yeast. These compounds are all potent competitive inhibitors of the enzyme and thus support the observation[3] that the naturally occurring 6-methyl-7-oxo-8-D-ribityllumazine[4] is a good competitive inhibitor.



(1)



(2)

R	K_i /M
Me	5×10^{-7}
Et	$2{\cdot}2 \times 10^{-6}$
$CH_2 \cdot Ph$	$2{\cdot}2 \times 10^{-6}$
CO_2H	$1{\cdot}0 \times 10^{-6}$
$CH_2 \cdot CH_2 \cdot CO_2H$	$1{\cdot}0 \times 10^{-5}$

The 7-oxolumazine (**2**, R = $CH_2.CH_2.CO_2H$) was synthesised specifically for use as a ligand in affinity chromatography. Sepharose 4B was activated with cyanogen bromide and subsequently reacted with di-(3-aminopropyl)-amine using known procedures[5] to give an amino-functionalised polymeric support. The ligand was coupled to this at pH 6·7 using 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride as the condensing agent.

The resulting blue fluorescent polymeric material was characterised by its u.v. spectrum (both in suspension and

after hydrolysis). This showed an attachment of $1{\cdot}2 \times 10^{-6}$ moles of oxolumazine per ml of settled polymer.

Application of a partially purified extract of riboflavin synthase (specific activity = 0·00089 units†) from baker's yeast to a column (70 × 10 mm) consisting of 5 ml of immobilised inhibitor resulted in complete retention of enzymic activity whereas a large amount of inactive protein passed straight through the column. Further inactive protein could be removed using 0·5M-KCl buffer. The enzyme (specific activity = 1·137 units) was then eluted biospecifically from the affinity column by the application of 10^{-2}M substrate (6,7-dimethyl-8-D-ribityllumazine). In a typical experiment a recovery of 87% of active material was achieved with a one-step purification factor of over 1000 fold.

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† Units used are moles ($\times 10^{-6}$) riboflavin produced per mg protein per min.

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