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CARBON-CARBON BOND FORMATION MEDIATED BY PAPAIN CHEMICALLY MODIFIED BY THIAZOLIUM SALTS

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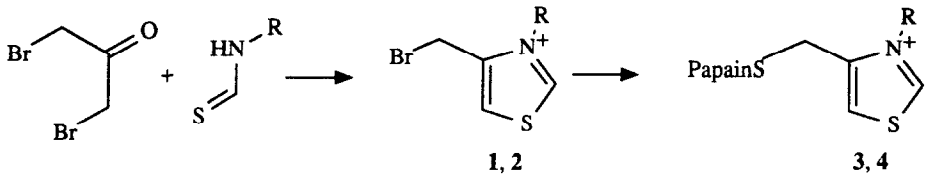
Summary: Alkylation of the active site cysteine with 2-bromomethyl- N-methyl- or 2-bromomethyl-N-benzyl-thiazolium bromide afforded a protein with no detectable protease activity but the ability to mediate carbon-carbon bond formation with or 6-oxoheptanal as substrate.

The importance of enzymes in chemical synthesis has been widely recognised both in the context of research and industrial production. A major challenge for bioorganic chemistry is the discovery of new protein based catalysts with predetermined catalytic activities. Site directed mutagenesis has provided a major technology for the modification of existing enzymes and some impressive successes have been achieved.[1] A second rational approach is to develop catalytic antibodies[2]. So far, however, interest in catalytic antibodies has focussed more on mechanistic aspects than on their potential as catalysts in chemical synthesis. Thirdly, existing enzymes with well-defined active sites can be modified by chemical reagents to introduce new reactivities[3]. Kaiser revived interest in this approach and in one case in which haemoglobin was modified, a protein with potentially useful synthetic activity was discovered[4]. If rational approaches to the production of new protein catalysts, including catalytic antibodies, are to be widely successful, it will be essential to have the ability to include chemical reactivity such as that provided by coenzymes beyond the range of aminoacid side chains. From the point of view of synthesis, carbon-carbon bond formation is crucial and accordingly we have investigated the modification of papain following Kaiser with thiazolium salts.

Suitable 2-bromomethylthiazolium salts for reacting with papain were prepared by the alkylation of an N-alkylthioformamide with 1,3-dibromoacetone in acetone solution at room temperature for three days (Figure 1: N-benzyl **1**, m.p. 182°C (decomp), 54%; N-methyl **2** m.p. 164-166°C (decomp) 39%). Papain was alkylated following Kaiser's procedures[5] in sodium phosphate buffer (pH 7.0, 0.01M) by the addition of three successive portions of the bromomethylthiazolium salt (each in five fold excess) at 0°C at five hour intervals; a five fold excess of cysteine was added before each of the second two additions. The modifed papains were separated from excess alkylating agent and cysteine by dialysis at 4°C against double distilled water and the solution freeze dried to afford the crude products (N-benzyl **3** 70%, N-methyl **4** 70%). The crude thiazolopapains retained about 35% of their peptidase activity. These proteins were purified by gel filtration on Sephadex G50 and residual unreacted papain was removed by affinity absorbtion on activated thiol

Sepharose[6] to afford proteins with no detectable peptidase activity using benzoylarginine ethyl ester as substrate. Alkylations of papain could not be effected with bromoethyl thiazolium bromides (R. Alijah, unpublished results).



1, 2 3, 4

Figure 1 **1, 3** R = CH_2Ph, **2, 4** R = Me

In preliminary studies we had shown that thiazolopapain **4** was able to decarboxylate pyruvic acid as shown by coupled enzyme assays for acetaldehyde and pyruvic acid[7]. To provide a more isolable product, we investigated the reaction of 6-oxoheptanal with **3** and **4** under conditions typical of preparative enzyme catalysed reactions in comparison with the simple thiazolium salts **1** and **2**. It was expected that cyclisation to 2-methylcyclopentanone derivatives would take place in the presence of modified papains. Reactions were carried out using the thiazolopapains **3** and **4** (4×10^{-7} mol) or the simple thiazolium salts (**1** 1.43×10^{-5} mol, **2** 1.64×10^{-5} mol) and 6-oxoheptanal (1.56×10^{-3} mol) in sodium phosphate buffer (pH 7.5 0.1 M)/acetonitrile 1:1 v/v at room temperature following the reaction by hplc. Time courses for the reactions are shown in figure 2. After 150 hours 6-oxoheptanal had been essentially comletely consumed in the reaction mediated by the N-benzylthiazolopapain **3** but was still present in the other reactions. The reactions were terminated and, the products extracted with ethyl acetate, and analysed by ^1H nmr at 250 MHz. In the case of reaction mediated by **3**, three components were clearly identifiable: unreacted 6-oxoheptanal (12% by integration), an expected product, 2-methylcyclohex-2-enone **5** (28%), and 7-hydroxytetradecane-2,8,13-trione **6** (60%)[8]. The N-methylthiazolopapapain **4** proved to be less effective in mediating the reaction affording **5** and **6** in 8% and 20% yields respectively. The simple thiazolium salts **1** and **2** were still poorer giving **6** only in 15% and 8% yields respectively.

The principal reaction mediated by the thiazolopapains thus appears to be a dimerisation of the substrate and a mechanism typical of thiazolium salt catalysis can be invoked (Figure 3). This result is reasonable if the active site pocket of papain is unable to accommodate both the covalently bonded cofactor and two molecules of substrate; preliminary molecular modelling experiments are consistent with this view.

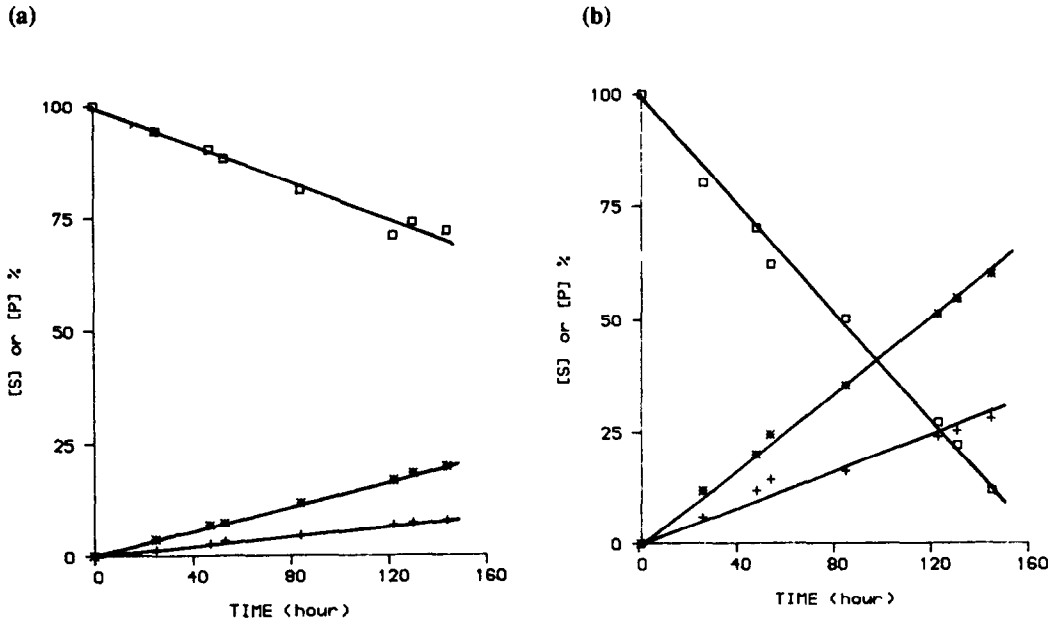


Figure 2 Time courses for reactions mediated by (a) N-methylthiazolopapain and (b) N-benzylthiazolopapain. □ [6-oxoheptanal], + [**5**], * [**6**] (%).

The above results are interesting from several points of view. Firstly, to our knowledge, these reactions (cyclisation and dimerisation) are the first examples of carbon-carbon bond formation by chemically modified enzymes. Secondly, they show that a protein bearing a coenzyme analogue as prosthetic group has some catalytic advantage over the free coenzyme analogue both in terms of overall yield and in being effective at much lower concentration (ca. 400 fold) and, in one case (**3**) in more than five fold better yield. In these reactions, the N-benzyl derivative **3** appears to be superior to the N-methyl analogue **4**, a pattern of behaviour that we have observed in related work with papain modified by N-alkylpyridinium salts; it is possible that this relates to the more favourable binding of the benzyl group than methyl in the hydrophobic binding site of papain[10]. Thirdly, two molecules of substrate apparently react mediated by the modified enzymes. It is not, however, possible to say whether the reactions occur fully within the active site pocket of papain or partially on the surface of the protein from the available information. Nevertheless the development of new protein catalysts for forming carbon-carbon bonds is viable target for research.

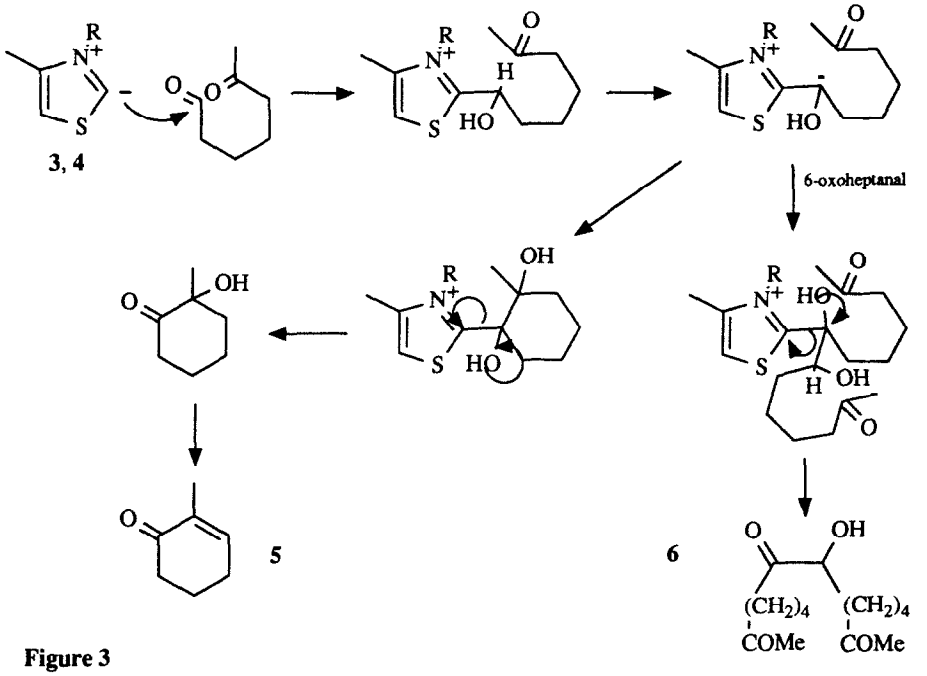


Figure 3

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