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AN UNUSUAL STABLE ADDUCT IN THE REACTION OF PYRUVATE WITH PAPAIN CHEMICALLY MODIFIED BY 3-ACETYL PYRIDINIUM AND QUINOLINIUM DERIVATIVES

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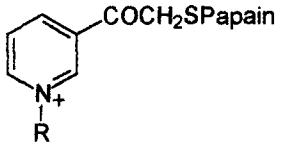
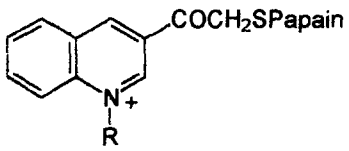
Summary: We have previously shown that electrophilic carbonyl compounds including ethyl pyruvate are *slowly* reduced by 3-acetyl-1,4-dihydropyridines attached to the active site thiol group of papain[1]. The mechanism of this reaction has been investigated using sodium 2-[^{13}C]-pyruvate as substrate. In contrast, reduction did not occur but the formation of an unexpected stable adduct assigned to the enol of the heterocyclic prosthetic groups to C-2 of pyruvate was observed.

The chemical modification of enzymes with non-natural cofactors is an established way of introducing new catalytic activity into chiral protein environments[1-3]. Recently, we showed that ethyl pyruvate was reduced in good yield but small enantiomeric excess by papain chemically modified by either N-ethyl- or N-benzyl-3-bromoacetylpyridinium salts (**1a, b**) in the presence of sodium dithionite[1].



1 a R = Et, **b** R = CH_2Ph



2 a R = Me, **b** R = CH_2Ph

The slowness of the reaction (48 h) for the reduction of such an electrophilic carbonyl group suggested that there might be a side reaction in which the dihydropyridines were behaving as enamines as has been known for some time in model reactions for NAD(P)H mediated reactions[4] (figure 1). To investigate this possibility, derivatised papains **1a, b** (8×10^{-7} mol) dissolved in sodium phosphate buffer (0.9 ml, 0.01M, pH 7 0) were reduced with sodium dithionite (1.6×10^{-5} mol) over 1.5 h and sodium 2-[^{13}C]-pyruvate ($1\ 6 \times 10^{-6}$ mol in 0.1 ml buffer) added The resonance of the 2-C of pyruvate (206 ppm) diminished over the course of 2h and a new peak at 89 ppm was observed. No such change was observed in a control experiment from which the modified papain was omitted. The new resonance at 89 ppm was stable for many hours and the formation of lactate as indicated by a resonance at 67 ppm was not observed.

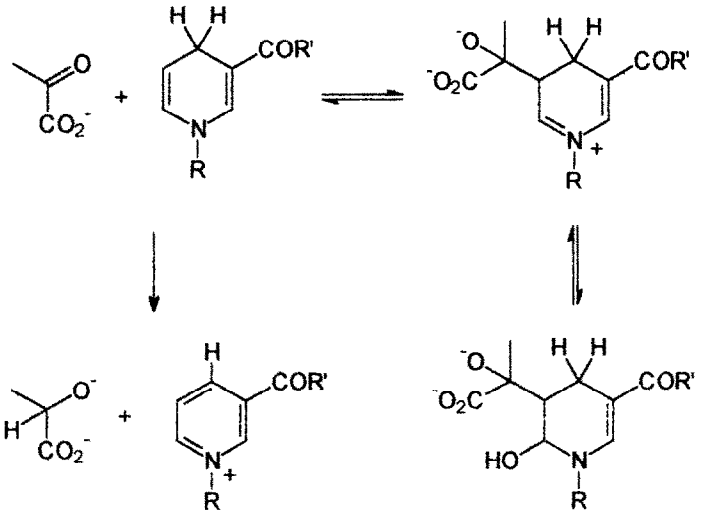


Figure 1 Enamine adduct formation in the reduction of electrophilic carbonyl groups by dihydropyridines[4]

The fact that the new resonance was 20 ppm downfield of that of lactate was not readily consistent with the shunt adduct in figure 1; an additional electronegative group attached to the labelled carbon was indicated To rule out the possibility of enamine like behaviour, we prepared the analogous 3-acetylquinolinium derivatives (**2a**, **b**) from the 2-methylquinoline chloral adduct via 3-acetylquinoline[5] followed by alkylation (**a** Me, **b** $PhCH_2Br$), bromination, and papain modification and purification as described previously[1]. The quinoline modified papains also underwent cycles of reduction with sodium dithionite to form the 1,4-dihydro derivative (λ_{max} 390 nm cf 340 nm for the dihydropyridines) and oxidation by air, oxidation of the dihydroquinolines (**2**) by air was significantly more rapid than that of the analogous dihydropyridines (**1**). N-Benzylquinolinium papain (**2b**) was reduced with sodium dithionite and incubated with 2-[^{13}C]-pyruvate as before An almost identical result was obtained with a new resonance at 90 ppm being observed.

Since it is highly improbable that the 3-acetyldihydroquinolines would behave as enamines in the manner indicated in figure 1, this result together with the chemical shift implicates the side chain in the adduct formation. ^{13}C-labelling has been widely used to demonstrate the presence of stable or metastable adducts in investigations of the mechanisms of action of peptidases For example, N-acetylphenylalaninal was shown to bind to chymotrypsin with the formation of a resonance at 94 ppm assigned to the hemiacetal adduct with the active site serine[6]. In a similar study of papain using N-acetylphenylalaninylgycinal[7], several species were observed in solution including the hydrate (88 2 ppm) and the active site thiol adducts (75.02 and 74 68 ppm) The new resonance observed in the experiments described above is therefore

exactly in the region expected for an oxygen adduct to the electrophilic carbonyl group of pyruvate. A further observation leads to the proposition of the mechanism shown in figure 2. In CD_3OD solution the ^1H nmr spectra of the bromoacetyl pyridinium and quinolinium salts showed evidence of ready enolisation through exchange of the protons of the α-methylene group for deuterium also the nucleophilic character of the ketone oxygen is promoted by donation of electrons from the ring nitrogen in the dihydro forms.

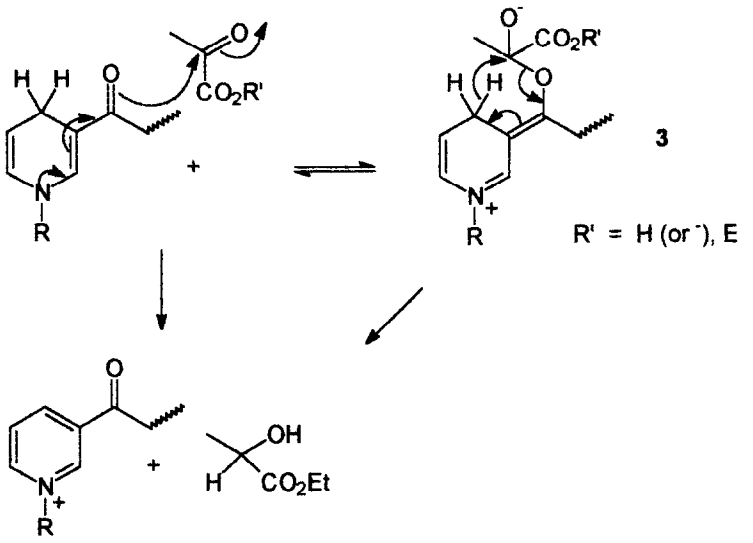


Figure 2. Proposed mechanism of formation of pyruvate adduct **3** and possible further reaction.

Hence we interpret the results of these experiments in terms of the addition of the oxygen atom of the prosthetic group to pyruvate affording the adduct **3**. In the case of pyruvate itself, this appears to be the end point of the reaction In the case of ethyl pyruvate, slow reduction could occur by infrequent direct hydride transfer or by slow electrocyclic fragmentation in a manner reminiscent of an old proposal for the mechanism of oxidation of alcohols by NAD(P) dependent dehydrogenases[8] that has found support in model reactions only[9-11]. The results described in this paper emphasise the difficulty transforming productively the catalytic reactivities of enzymes by chemical modification.

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