Approaches to Chemically Modified Enzymes As Synthetic Catalysts

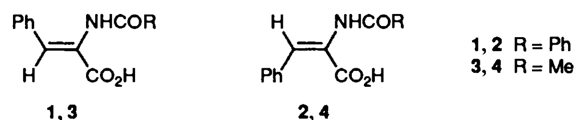
David J. Aitken, Renate Alijah, Samuel O. Onyiriuka, Colin J. Suckling,* Hamish C. S. Wood and Limin Zhu
Department of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, G1 1XL, UK

Attempts to introduce new catalytic activities of potential use in synthetic transformations into enzyme active sites are described. Substitution of the naturally occurring zinc in carboxypeptidase A by several metals known to catalyse hydrogenation was investigated; a new protein characterised as a rhodium carboxypeptidase was isolated but it failed to show activity as a hydrogenation catalyst for the reduction of a series of dehydroamino acid amides. Horse liver alcohol dehydrogenase was investigated for its potential to act as an oxygen transferase *via* Lewis acid catalysis. A series of cyclohexenyl and phenylribosides together with new alkenyl(arenyl)oxymethylenoxyethanols was prepared for evaluation as substrates; in the course of this study, novel neighbouring group participation by the oxygen atom of a chloromethyl ether was observed. Although the binding of potential oxygen acceptors (alkenes and aromatic compounds) and oxygen donors (hydrogen peroxide and alkyl hydroperoxides) was demonstrated, oxygen transfer did not occur with the combinations investigated. In contrast to the failure of the above metalloenzymes to catalyse new reactions, papain modified at the active site sulfhydryl group by thiazolium salts and pyridinium salts was shown to exhibit reactions characteristic of the covalently attached cofactor. Thus the thiazolopapains acted as decarboxylation catalysts for pyruvate and the pyridinopapains could be reduced to dihydropyridines which reduced electrophilic carbonyl substrates with small enantiomeric excess.

The importance of enzymes in chemical synthesis has now been widely recognised [1] and the success of such biological catalysts has raised questions of the identification and design of new protein-based catalysts with predetermined catalytic activities. There are several possible approaches to selective catalysis. New enzymes may be isolated from nature,[2] new selective chemistry may be discovered,[3] new catalytic proteins may be obtained through antibody technology.[4] A fourth approach, the chemical modification of enzymes has been described in detail by Kaiser with respect to several modifications of papain [5] but only in one case, in which the modified protein was haemoglobin, was a protein with a potentially useful synthetic activity discovered.[6] The essence of Kaiser's strategy was to introduce a cofactor into the active site region of the protein through alkylation of a cysteine residue by flavins that can function as redox catalysts. In this way, for example, a peptidase was transformed into an oxidoreductase. Modification of thiol-containing proteins is restrictive in the chemistry that can be introduced. Further, if the full potential of the new technology of catalytic antibodies is to be realised, it will be necessary to introduce cofactor groups into the antibodies,[7–9] and especially non-natural cofactors. For these reasons, we became interested in modifying proteins to introduce new and potentially useful synthetic reactivities.

Modification of Metalloenzymes.—Several enzymes are known to catalyse reactions other than that for which they evolved. Carboxypeptidase A, (CPA) a peptidase, for example, catalyses β-elimination of suitable substrates [10] as well as proton exchange in non-hydrolysable substrates and the metal-exchanged copper-CPA catalyses the oxidation of ascorbic acid.[11] Horse liver alcohol dehydrogenase catalyses carbon–oxygen bond cleavage in susceptible substrates [12] and also has been shown to have a peroxidase activity in the presence of hydrogen peroxide and NADH.[13] A limited number of applications of CPA to synthesis have been described [14] but HLADH has been widely used in synthesis.[15] Both of these enzymes are zinc metalloproteins and we therefore selected them for study.

Carboxypeptidase A. With CPA, the reaction chosen was hydrogenation. To effect hydrogenation, it would be necessary to replace the zinc ion at the active site with a metal ion known to act as a hydrogen transfer catalyst. Metal exchange in CPA has been known for many years [16] but of the ions exchanged, only Ni^{2+} might conceivably be expected to show any catalytic activity in hydrogenation. In this work, the exchange of rhodium, ruthenium, and palladium as new substitutions were investigated together with known substitutions of cobalt and nickel. Following work on homogeneous catalysis and the peptidase activity of CPA, dehydroamino acids **1–4** were prepared as substrates by known methods [17–19] (see Experimental section for details).



Metal exchange procedures were based upon the method of Latt and Vallee.[20] A solution of bovine pancreatic CPA (Sigma Chemical Co.) in aqueous saline buffer at 0 °C was dialysed repeatedly against 1,10-phenanthroline and then against ligand free buffer to afford the apo-enzyme. The appropriate metal dication was then introduced by dialysis of the apo-enzyme solution against an aqueous solution of the metal dication. Finally, the reconstituted enzyme was crystallised by dialysis against low ionic strength buffer. The Co- and Ni-CPAs had spectroscopic properties in agreement with that described in the literature.[20,21] Neither these proteins nor the native Zn-CPA were found to catalyse the hydrogenation of any of the dehydroamino acids **1–4** (40 fold excess) in aqueous saline buffer at atmospheric pressure and ambient temperature.

The lack of reactivity of these proteins was not surprising and attempts were made to exchange metals with a broader pedigree of ability to catalyse hydrogenation reactions. Experiments to prepare Ru-CPA and Pd-CPA were disappointing. Dialysis of apo-CPA against Ru^{III} or Pd^{II} chlorides led to complete denaturation of the enzyme. First experiments with rhodium suggested that a similar denaturation of the protein had taken place but the denaturation was shown only to be partial. Dialysis against Rh^{III} chloride led to the formation of a pale pink precipitate in the dialysis tube and a further sample of protein was crystallised by dialysis against a weak electrolyte. A solution of protein was obtained by redissolving the precipitate in saline buffer and characterisation of the putative Rh-CPA attempted.

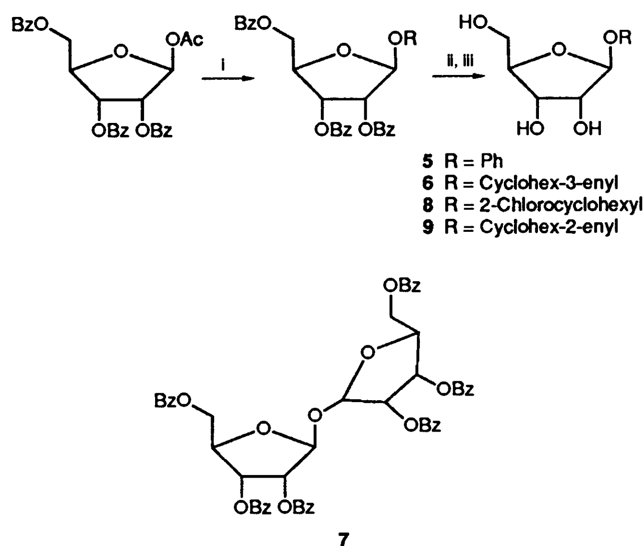
The solution of Rh-CPA was shown to contain equimolar CPA and Rh by atomic absorption spectroscopic analysis. However dialysis of a fresh sample of Rh-CPA against three changes of metal-free buffer led to a sample that contained only 15 mol% of rhodium compared with protein. Evidently Rh-CPA is prone to dissociation, an effect that has been observed with other metal ions.[16] The 1:1 Rh-CPA had very low peptidase activity, not more than 4% of the native activity; such a low residual activity could be due to traces of native enzyme. Hence it could be that Rh-CPA is inactive as a peptidase. The electronic spectrum of Rh-CPA showed a weak maximum at 490 nm (ε 221 mol^{-1} dm^3 cm^{-1}). The preferred geometry for Rh^{III} complexes is six coordinate octahedral,[22] and a six coordinate structure could be attained in the coordination site of CPA despite distortions from regular geometries.[23] The observed spectrum has similarities with those reported for hydrated Rh^{III} chloride complexes.[24] It can therefore be concluded that the protein obtained was indeed a Rh-CPA.

Tests of the ability of Rh-CPA to mediate hydrogenation of **1**–**4** were carried out as outlined for Co- and Ni-CPA. Although a distinct colour change from red to yellow was observed when hydrogen was absorbed by the solution, no evidence for hydrogenase activity was obtained by analysis of the solutions by HPLC.

The failure to observe hydrogenase activity in any case could be due to the intrinsic reactivity of the metal ions in the coordination environment of CPA or possibly to insufficiently strong binding of the selected substrates to CPA. To evaluate the latter possibility the kinetics of inhibition of hydrolysis of hippurylphenylalanine by dehydroamino acids **1**–**4** were investigated. Both the *Z*-isomers (**1** and **3**) were found to be weak inhibitors ($K_i \geqslant 10^{-3}$ mol dm^{-3}) and the kinetic mechanism of inhibition could not be diagnosed with certainty from Dixon plots. On the other hand, the *E*-isomers were competitive inhibitors binding similarly strongly to the substrate (**2** $K_i = 1.3 \times 10^{-4}$ mol dm^{-3}; **4** $K_i = 5.5 \times 10^{-4}$ mol dm^{-3}). If the interaction between Rh-CPA and either **2** or **4** was favourably oriented with respect to the metal and the chemical reactivity of the coordinated Rh was suitable, hydrogenation could conceivably occur. However, it is difficult to ascertain that a favourable interaction can take place. Low level molecular modelling experiments suggested that an appropriate conjugation of double bond and metal ion may not be possible. The failure of hydrogenation could thus be due either to the intrinsic chemistry of the M-CPAs or to the substrates selected for the initial study. Further work will be necessary to distinguish between these possibilities.

Horse liver alcohol dehydrogenase. The feature of HLADH chemistry that we wished to explore was the Lewis acid-activity of the catalytic zinc ion. The potential of this enzyme for inducing additional chemical reactions is evident from several reports of non-dehydrogenase activity including C–O bond cleavage,[12] aldehyde dismutase,[25] *C*-nitroso reductase[26] and esterase[27] activities. In particular, HLADH has been reported to act as a peroxidase in the presence of NAD[13] and the final product of oxidation was identified as a decomposition product of NAD in which the nicotinamide ring had been degraded. We explored the possibility of extending this reaction to electrophilic hydroxylation of aromatic compounds and the oxidation of alkenes. Chemical methods for the latter have reached a high degree of sophistication through the Sharpless reaction[3] but hydroxylation of aromatic compounds under mild conditions is still a challenging goal.

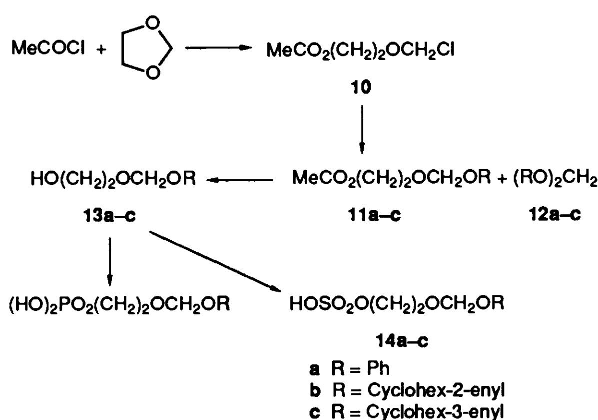
To approach this goal, we investigated *tert*-butylhydroperoxide as oxygen donor and cyclohexene and anisole as first substrates. Not surprisingly, no oxidation was observed because there is little in these simple substrates for recognition by HLADH. Therefore we synthesised a number of glycosides and analogues with phenol and cyclohexenes as aglycones in an attempt to take advantage of part of the coenzyme-binding pocket to induce reaction. The synthesis of a phenyl riboside **5** was accomplished as shown in Scheme 1[28] and the same



Scheme 1 *Reagents:* i, $SnCl_4$, CH_2Cl_2, ROH; ii, NaOMe, MeOH; iii, KOH, MeOH for **9** only

sequence was successful for cyclohex-3-enol to give **6**. However, when the aglycone was cyclohex-2-enol, a complex mixture of products was obtained from which 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl 2,3,5-tri-*O*-benzoyl-β-D-ribofuranoside **7** was isolated. To circumvent this unexpected side reaction, the aglycone *trans*-2-chlorocyclohexanol **8** was prepared[29] and the required cyclohexene **9** obtained by elimination (Scheme 1). The ribosides were purified by chromatography and the configuration established as β by NMR spectroscopy.

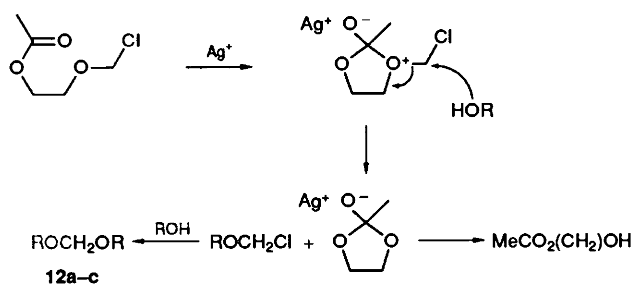
In addition to the three ribosides **5**, **6** and **9**, a series of acyclic analogues was prepared using 2-(chloromethoxy)ethyl acetate[30] (**10**, Scheme 2). Treatment of **10** with lithium cyclohex-2-enolate afforded mostly the corresponding acetate with less than 10% of the required substitution product **11b**. Under milder conditions,[31] using silver carbonate, a mixture of the required product **11b** together with the dicyclohexenoxymethane **12b** was obtained. Similar reactions occurred with the 3-ene isomer affording **11c** and **12c**. Although the yields were not high, sufficient material was obtained from these reactions for attempted phosphorylation and use in enzyme assays. Phosphorylation was attempted with the more readily available phenyl glycoside analogue **13a**. Compound **13a** was phosphorylated with diphenylphosphoryl chloride but the selective hydrolysis of the phenyl phosphate esters was difficult although some of the required product was obtained using lithium hydroxide to remove the first phenoxy group followed by



Scheme 2

barium hydroxide under vigorous conditions. Alternatively, dianilidophosphoryl chloride[32] afforded the expected ester but cleavage with freshly prepared isoamyl nitrite in the presence of pyridine and acetic acid led only to an impure sample of the required compound. Rather than test the properties of HLADH with impure samples, the sulfates of the three glycoside analogues **14a–c** were prepared using freshly prepared pyridine–sulfur trioxide complex. The initially formed pyridinium salts were converted into the corresponding sodium salts by ion exchange chromatography and the products stored frozen in pH 9 aqueous buffer to avoid acid catalysed decomposition to phenol or cyclohexenols.

As noted above, in reactions of 2-(chloromethoxy)ethyl acetate with alcohols in the presence of silver carbonate, a significant by-product was the bis(alkoxy)methane. The reaction did not occur with phenol but bis(alkoxy)methanes were also obtained with benzyl alcohol and cyclohexanol as substrates.[33] These products were not formed in the absence of 2-(chloromethoxy)ethyl acetate and underwent no further reactions when treated with additional reagent and silver carbonate under the reaction conditions. Hence the acetal carbon of the bis(alkoxy)methanes probably arises from 2-(chloromethoxy)ethyl acetate and a plausible mechanism involving a cyclic oxonium ion is shown in Scheme 3. This



Scheme 3

mechanism may also offer an explanation for the complexity of the reaction with phenol; silver carbonate could be considered as generating the equivalent of a powerful electrophile ($ClCH_2^+$) which could be capable of substitution reactions attacking the phenol ring. Although neighbouring group participation in cyclic oxonium intermediates is well known[34] this is to our knowledge the first report of the participation of a chloromethyl ether oxygen.[33]

The six substrates prepared as described above were used to test the ability of HLADH to act as an oxygen transferase from *tert*-butyl hydroperoxide or hydrogen peroxide. Typically, the enzyme was incubated in buffered aqueous solution at room temperature with equivalent amounts of substrate and oxygen donor. Experiments were carried out on an analytical scale in two series, one in the absence and one in the presence of adenosine monophosphate (AMP) which would occupy a further part of the coenzyme binding site and hence might enhance binding of the substrates. To determine the products from the oxidation of the phenyl glycoside **5** and analogue **14a**, the reaction products were heated with concentrated acid and the phenolic products determined by HPLC. Using these methods, no hydroxylated products were detected. Analysis of the possible products of epoxidation of cyclohexenyl ethers was more complex because of the lack of a chromophore for HPLC and the ability of the epoxides to ring open under the conditions used to cleave the acetals. The analysis for oxidation was therefore based upon the detection of cyclohexane-1,2,3- and 1,2,4-triols; NMR spectroscopy was found to be the best analytical method. To isolate the alcohols, products from the test reactions were passed through a mixed bed ion exchange column to remove buffer salts, enzyme, and AMP and the eluate evaporated to dryness. Although the presence of cyclohexenols was detected, no evidence was obtained for the presence of oxidation products.

The negative results obtained could arise from either the inability of HLADH to react with the peroxides used or from unfavourable binding of the selected substrates. Since no products were obtained when hydrogen peroxide was used and the degradation of NAD has been reported[13] it is unlikely that the intrinsic chemical reactivity of HLADH is the problem. To shed some light on the binding of the substrates, their properties as enzyme inhibitors were examined. *tert*-Butylhydroperoxide was shown to be a competitive inhibitor of HLADH (K_i 6.75×10^{-4} mol dm^{-3}) with respect to ethanol, and hydrogen peroxide exhibited mixed inhibition kinetics (K_i 4.2×10^{-2} mol dm^{-3}); previous work has shown that ethanol is competitive with hydrogen peroxide.[13] Thus the oxidising agents appear to be competent for the required chemistry since the substrates were designed to occupy part of the coenzyme binding site, their ability to compete with NAD^+ was the appropriate test. Using the phenyl glycosides and analogues as representative examples, we found that phenyl β-D-ribofuranoside **5** did not inhibit at concentrations up to 5 mmol dm^{-3}. The sodium salt of 2-(phenoxymethoxy)ethyl sulfate **14a** was a weak inhibitor but the affinity was too weak to identify conclusively the nature of the inhibition; the most probable interpretation of the results suggested non-competitive inhibition with respect to NAD^+ (K_i 7 mmol dm^{-3}). Computer graphics studies also suggested that the binding of these compounds to HLADH in the presence of an oxidising agent might be difficult and indicated that the acetals selected for study could not present the carbocylic rings that were targets for oxidation in the pyridinium ring binding region because of severe steric interactions in all conformations. It did appear, however, that a compound lacking the exocyclic acetal oxygen might indeed fit and suggested that *C*-nucleosides and their analogues might be suitable substrates. This possibility is supported by results reported after this work was carried out that show that HLADH is not tolerant of truncated ribose residues but that nicotinamide riboside is an effective coenzyme in the presence of AMP.[35] The possibility still remains that HLADH could act as an oxygen transferase for ribose *C*-nucleosides in the presence of AMP. Clearly such a process would not be of general synthetic interest but, if it occurred, the knowledge would assist the development of novel catalytic proteins.

Modification of Papain.—Several years ago Kaiser recognised the potential of the reactive thiol at the active site of papain for the introduction of covalently bound prosthetic groups that would introduce new chemical reactivity in place of peptide

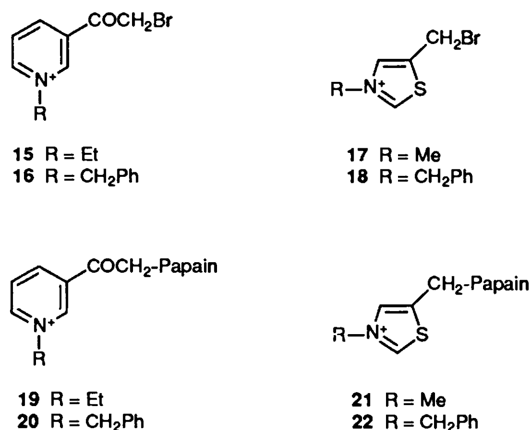
Table 1 Purification of modified papains

Sample	Step	Activity[a] (%)	Yield[b] (%)
19	1	29.3	75
	2	7.8	70
	3	0	69
20	1	23.0	75
	2	9.5	83
	3	0	76
21	1	35.9	70
	2	4.7	63
	3	0	70
22	1	35.0	70
	2	14.9	70
	3	0	50

[a] Peptidase activity measured with benzoylarginine ethyl ester. [b] Yield based upon weight of protein recovered.

hydrolysis.[5] His studies concentrated the properties of so-called 'flavopapains' in which flavins had been introduced and he was able to demonstrate catalysis of hydrogen transfer and oxidation of thiols. We wished to discover whether such a device could be extended to proteins with greater synthetic potential and we selected the introduction of nicotinamide and thiazolium cofactor analogues, to give pyridinopapains and thiazolopapains, as test cases.

Preparation of modified papains. The modified proteins were obtained by alkylation of papain with active halogen derivatives of the respective heterocyclic systems (Scheme 4). Thus 3-



acetylpyridine was alkylated with ethyl bromide and benzyl bromide and the pyridinium salts brominated with bromine in chloroform–ether at 0 °C to afford the alkylating agents **15** and **16**. 5-Bromomethyl-*N*-methylthiazolium bromide **17** was prepared by treating 1,3-dibromoacetone with *N*-methylthioformamide in acetone and the corresponding *N*-benzylthiazolium salt **18** prepared from *N*-benzylthioformamide. These reagents were used to modify papain under conditions similar to those used by Kaiser.[36] Papain was treated with successive portions of the alkylating reagent in pH 7 phosphate buffer at 0 °C for 18–24 h. When peptidase activity had reached a minimum as determined by a standard assay with benzoylarginine ethyl ester or benzoylarginine 4-nitroanilide as substrate, the solution of protein was freeze dried. The crude modified papains were purified in three stages by dialysis to remove residual alkylating reagent, by gel filtration to concentrate the relevant protein fraction, and finally by treatment with thiol-activated Sepharose to remove any residual unmodified papain. The progress of the purification is

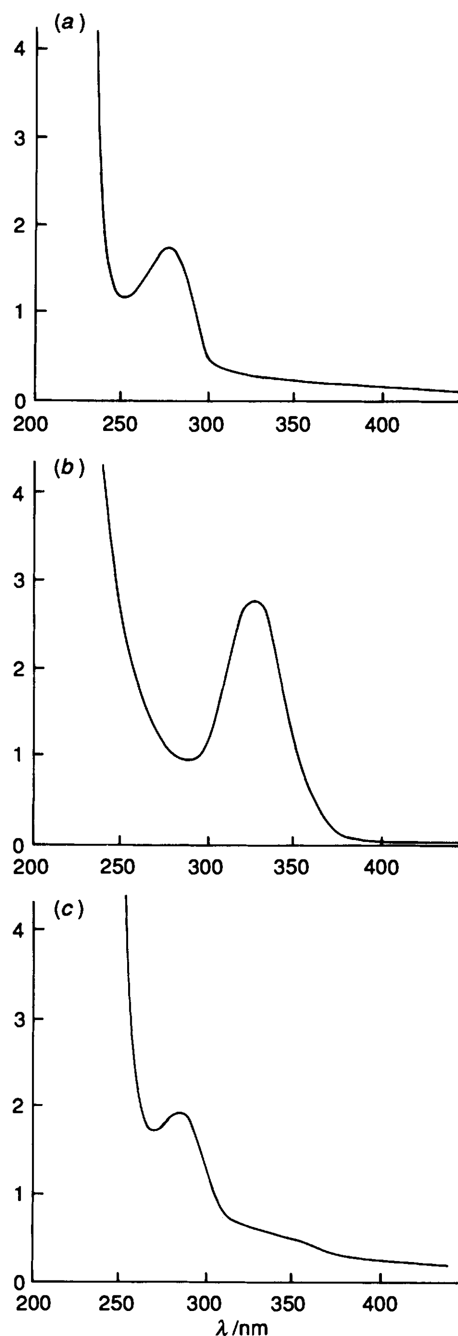


Fig. 1 Oxidation–reduction behaviour of pyridinium salt modified papains: (*a*) oxidised form; (*b*) 1,4-dihydro form after reduction with $Na_2S_2O_4$; (*c*) oxidised form after reduction of ethyl pyruvate

summarised in Table 1. The four alkylating agents showed kinetic properties typical of active site directed irreversible inhibitors of enzymes including time and concentration dependent inactivation. The pyridinium salts **15** and **16** bound more tightly to the papain (K_i **19** 0.016, **20** 0.010 mmol dm^{-3}) than the thiazolium salts (K_i **21** 1.80, **22** 1.00 mmol dm^{-3}).

Reactions of modified papains. Thiazolopapains **21** and **22** were examined for their ability to catalyse a typical thiamine coenzyme reaction, namely decarboxylation of pyruvic acid. In order to assay the reaction, both the product and reactant was coupled to an NADH dependent dehydrogenase so that the course of the reaction was monitored by the change in absorbance of NADH at 340 nm. Thus the formation of acetaldehyde was detected using horse liver alcohol dehydrogenase and the consumption of pyruvic acid by lactate

Table 2 Reduction mediated by pyridinopapains

Sample	Substrate	Recycling agent	Time (h)	Yield (%)	e.e. (%)
19	Ethyl pyruvate	$Na_2S_2O_4$	48	6	—
20	Ethyl pyruvate	$Na_2S_2O_4$	48	96	—
19	Ethyl pyruvate	*N*-Ethyl-1,4-dihydronicotinamide	51	100	2.6(*R*)
—	Ethyl pyruvate	*N*-Ethyl-1,4-dihydronicotinamide	51	75	0
19	α,α,α-Trifluoroacetophenone	*N*-Ethyl-1,4-dihydronicotinamide	76	15	—
20	α,α,α-Trifluoroacetophenone	*N*-Ethyl-1,4-dihydronicotinamide	47	35	7.5(*S*)
—	α,α,α-Trifluoroacetophenone	*N*-Ethyl-1,4-dihydronicotinamide	71	28	0
19	α,α,α-Trifluoroacetophenone	*N*-Benzyl-1,4-dihydronicotinamide	69	71	14.2(*R*)
20	α,α,α-Trifluoroacetophenone	*N*-Benzyl-1,4-dihydronicotinamide	69	55	11.6(*R*)
—	α,α,α-Trifluoroacetophenone	*N*-Benzyl-1,4-dihydronicotinamide	69	55	0

dehydrogenase. Although it was possible to demonstrate decarboxylase activity in this way the thiazolopapains did not catalyse the synthesis of acetoins from acetaldehyde or benzaldehyde and their detailed study was therefore discontinued.

The value of nicotinamide-dependent dehydrogenases in preparative organic chemistry is well established[15] and the intention of this study was not simply to replicate such chemistry but to extend the range of cofactors available and to investigate the behaviour of cofactors in new chiral environments. The first experiments investigated the reducibility of the artificial cofactor by sodium dithionite; at pH 8, the pyridinopapains were both reduced to the corresponding dihydropyridines as shown by the absorption at 336 nm (Fig. 1). The reduced pyridinopapains were then incubated in the presence of sodium dithionite with ethyl pyruvate on a preparative scale. Under these conditions, after two days reaction, essentially complete conversion of ethyl pyruvate to ethyl lactate (96%) was accomplished by benzylpyridinopapain **20** but the conversion mediated by ethylpyridinopapain **19** was only 6% (Table 2). Having identified that catalytic oxidation was occurring, a series of further potential substrates was investigated this time using *N*-ethyl-1,4-dihydronicotinamide as the recycling agent. Neither benzaldehyde nor cyclohexanone was reduced; the only accepted substrates were those with strongly electrophilic carbonyl groups such as ethyl pyruvate and α,α,α-trifluoroacetophenone. Both of these compounds also underwent reduction by dihydropyridines in the absence of enzymes and experiments with the pyridinopapains were carried out in parallel with control reactions in which the modified papain was omitted. For trifluoroacetophenone, both ethyl- and benzyl-pyridinopapains mediate reduction as does the recycling agent *N*-benzyl-1,4-dihydronicotinamide. Complete reduction was not observed and the rates of reaction were slow. Evidence that the reactions, at least in part, take place at the site of the modified papains comes from the fact that the products isolated from these reactions showed small but significant optical rotations; an enantioselective reaction would, of course, be expected at the active site of a protein. When ethyl pyruvate was used as substrate, in the presence of a dihydropyridine recycling agent, reaction was not greatly enhanced over the control experiment without *N*-benzylpyridinopapain and a very small enantiomeric excess was observed in the presence of protein.

Discussion

Although some evidence for reaction at the active sites of pyridinopapains has been obtained, the results described in this paper together show the difficulty in introducing new chemistry into the highly evolved active sites of enzymes. Yet, if protein catalysis is not to be limited to reactions catalysed by nature, or to entropic sinks and hydrolysis reactions such as those mediated by catalytic antibodies[4] it is essential that methods of introducing new chemical reactivity into proteins be discovered. In the case of catalytic antibodies, it has been possible to replicate some functions of naturally occurring cofactors such as flavins[37] and pyridoxal[9] but a radically novel chemical reactivity has not been introduced. It has been said that it is easier to inhibit enzymes than to design new ones; so far, the greatest success has been with genetically modified enzymes[38] with the discovery of new natural enzymes belonging to recognised classes.

All of our investigations had some basis in the known properties of the enzymes studied. The required modifications were in most cases readily accomplished. However even when the planned chemical reactivity was demonstrated or known, there were problems either with the binding of substrates (HLADH) or side reactions of the cofactor (pyridinopapain). The successful development of a new catalytic protein will therefore require a much broader treatment of the whole process of catalysis including binding equilibria and the robustness of the system.

Experimental

NMR spectra were recorded on a Perkin-Elmer R32 spectrometer (90 MHz) or Bruker WM250 (250 MHz). *J* values are given in Hz. $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}.

Preparation of substrates and modifying agents. The following compounds were prepared following published procedures: (*E*)-2-phenyl-4-phenylmethylenoxazol-5(4*H*)-one,[18] (*E*)-2-acetamido-3-phenylprop-2-enoic acid **3**,[19] (*Z*)-2-phenyl-4-phenylmethylenoxazol-5(4*H*)-one,[17] (*Z*)-2-benzamido-3-phenylprop-2-enoic acid **2**,[17] (*E*)-2-benzamido-3-phenylprop-2-enoic acid **1**,[19] methyl 2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl chloride,[39] 2-(chloromethoxy)ethyl acetate.[30]

Phenyl 2,3,5-tri-O-Benzoyl-β-D-ribofuranoside.—A solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose (2.52 g, 5.0 mmol) in dichloromethane (50 cm^3) was cooled to 0 °C and tin(IV) chloride (0.6 cm^3, 1.34 g, 5.1 mmol) was added. The mixture was kept at 0 °C for 15 min, then a solution of phenol (0.47 g, 5.0 mmol) in dichloromethane (2 cm^3) was added. Moisture was excluded during these operations. After 4 h at 0 °C, the mixture was poured into saturated aqueous sodium hydrogen carbonate (150 cm^3). The organic layer was collected, and combined with dichloromethane washings (2 × 50 cm^3) of the residual aqueous layer. The organic extracts were washed with water (50 cm^3), dried ($MgSO_4$), and the solvent evaporated under reduced pressure to leave a viscous oil (2.8 g) which solidified on standing for 2 days. The solids were triturated with cold ethanol, then recrystallised from the same solvent to give white needles (1.04 g, 39%), m.p. 132–133 °C (lit.,[28] 132–133 °C) (Found: C, 71.35; H, 4.8. Calc. for $C_{32}H_{26}O_8$: C, 71.35; H, 4.85%); $[\alpha]_D^{22}$ − 8.6 (*c* 0.92 in acetone) [lit.,[28] $[\alpha]_D^{20}$ − 7.8 (*c* 0.94 in acetone)]; ν_{max}(KCl)/cm^{-1} 3060, 3030, 1710, 1695, 1685, 755, 710 and 690; δ_H(250 MHz; $CDCl_3$) 4.54 (1 H, dd, *J* 11.9 and

4.6, 5'-H), 4.72 (1 H, dd, *J* 11.9 and 4.4, 5-H), 4.83 (1 H, ddd, *J* 4.6, 4.4 and 7.0 4-H), 5.91 (1 H, s, 1-α-H), 5.93 (1 H, d, *J* 4.8 2-H), 6.04 (1 H, dd, *J* 4.8 and 7.0 3-H), 6.97–7.06, 7.22–7.62 and 7.90–8.06 (20 H, 3 m, PhO and $PhCO_2$).

Cyclohex-3-enyl 2,3,5-tri-O-Benozyl-β-D-ribofuranoside.—The previous procedure was followed exactly, using cyclohex-3-enol (0.49 g, 5.0 mmol) instead of phenol. When the reaction was quenched in saturated aqueous sodium hydrogen carbonate solution the mixture was stirred for 10 min while the colouration disappeared from the organic layer and a white suspension developed in the aqueous layer. The crude product was a syrup (2.85 g). Flash chromatography using 2% ethyl acetate in benzene yielded the *title compound* as a clear viscous syrup (1.08 g, 40%) (Found: C, 70.8; H, 5.65. $C_{32}H_{30}O_8$ requires C, 70.85; H, 5.55%); $[\alpha]_D^{23}$ + 27.0 (*c* 0.57 in acetone); ν_{max}(film)/cm^{-1} 3130, 3050, 1715, 1600, 1580 and 710; δ_H(250 MHz; $CDCl_3$) 1.55–1.72, 1.88–2.18, 2.26–2.44 (6 H, 3 m), 3.97 (1 H, m, 1-H), 4.51–4.59 (1 H, m, 5'-H), 4.65–4.75 (2 H, m, 4- and 5-H), 5.43, 5.45 (1 H, s, 1-H diastereoisomers), 5.51–5.57, 5.61 (2 H, 2 m, CH=CH), 5.65 (1 H, d, 2-H), 5.86, 5.88 (1 H, 2 dd, 3-H diastereoisomers), 7.29–7.61, 7.87–7.91 and 7.99–8.07 (15 H, 3 m).

Reaction of 1-O-*Acetyl*-2,3,5-*tri*-O-*benzoyl*-β-D-*ribofuranose with Tin*(IV) *Chloride and Cyclohex-2-enol*.—A solution of 1-*O*-acetyl-2,3,5-tri-*O*-benzyl-β-D-ribofuranose (504 mg, 1.0 mmol) in dichloromethane (10 cm^3) was cooled to 0 °C and tin(IV) chloride (0.12 cm^3, 0.27 g). The mixture was kept at 0 °C for 15 min, then a solution of cyclohex-2-enol (64) (98 mg, 1.0 mmol) in dichloromethane (1 cm^3) was added. After 1 h at 0 °C the mixture was poured into saturated aqueous sodium hydrogen carbonate (30 cm^3) and stirred as a series of colour changes took place through red, violet and blue to pale green, while white solids developed in the aqueous phase. The organic layer was collected and combined with further dichloromethane washings (2 × 10 cm^3) of the aqueous layer. Organic extracts were washed with water (2 × 10 cm^3) then dried ($MgSO_4$). The pale green solution was evaporated under reduced pressure to give solids (540 mg). TLC analysis (silica; 10% ethyl acetate in $CHCl_3$) showed a long streak from R_f to 1.0, plus components R_f 0.55, 0.49 and 0.18. Flash chromatography using 10% ethyl acetate in chloroform as eluent did not give efficient separation of components; by crystallisation from ethanol and re-crystallisation, a single component was isolated and identified as 2,3,5-*tri*-O-*benzoyl*-β-D-*ribofuranosyl* 2,3,5-*tri*-O-*benzoyl*-β-D-*ribofuranoside* (108 mg, 24%), m.p. 144–145 °C (lit.,[40] 144–145 °C) (Found: C, 68.9; H, 4.65. Calc. for $C_{52}H_{42}O_{15}$: C, 68.85; H, 4.65%); δ_H(250 MHz; $CDCl_3$) 4.57 (1 H, dd, *J* 12.8 and 6.6, 5'-H), 4.73–4.78 (2 H, m, 4- and 5-H), 5.68 (1 H, d, *J* 4.8 2-H), 5.71 (1 H, s, 1-α-H), 5.88 (1 H, dd, *J* 4.8 and 6.6, 3-H), 7.25–7.60 and 7.85–7.99 (15 H, 2 m).

trans-2-*Chlorocyclohexyl* 2,3,5-*Tri*-O-*benzoyl*-β-*ribofuranoside*.[29]—The procedure for the cyclohex-3-enyl analogue was followed exactly, using *trans*-2-chlorocyclohexanol (0.67 g, 5.0 mmol) instead of cyclohex-3-enol. The crude product was a clear syrup (4.3 g). Flash chromatography using 2% ethyl acetate in benzene yielded the *title compound* (1.28 g, 44%), $[\alpha]_D^{24}$ + 17.0 (*c* 0.91 in $CHCl_3$) [lit.,[29] $[\alpha]_D^{25}$ + 22 (*c* 0.9 in $CHCl_3$)]; ν_{max}(film)/cm^{-1} 3060, 3025, 1720, 1600, 1580, 710 and 680; δ_H(250 MHz; $CDCl_3$) 1.19–1.47 (3 H, m), 1.58–1.75 (3 H, m), 2.12–2.23 (2 H, m), 3.57–3.95 (2 H, m, 1- and 2-H), 4.54, 4.65–4.79 (3 H, dd and m, 4-H and 5-H_2), 5.44, 5.55 (1 H, 2 s, 1-α-H diastereoisomers), 5.66, 5.74 (1 H, 2 d, *J* 4.8 and *J* 5.0, 2-H diastereoisomers), 7.28–7.61 and 7.85–8.09 (15 H, 2 m).

Phenyl β-D-*Ribofuranoside* **5**.—A solution of sodium methoxide was prepared by dissolved sodium (40 mg) in dry methanol (5 cm^3). An aliquot (3 cm^3) of this solution was added to a suspension of phenyl 2,3,5-tri-*O*-benzoyl-β-D-ribofuranoside (0.98 g, 1.8 mmol) in methanol (50 cm^3). The mixture was protected from moisture, and stirred at ambient temperature for 12 h, dissolution having been achieved after only several minutes. The clear solution was treated with water (10 cm^3) then saturated with carbon dioxide. Evaporation of the solvent under reduced pressure left solids, which were digested in a small amount of acetone, and separated from inorganic salts by filtration. The filtrate was evaporated and the product recrystallised from benzene as white needles (0.32 g, 78%), m.p. 106–107 °C (lit.,[28] 106–107 °C) (Found: 58.1; H, 6.05. Calc. for $C_{11}H_{14}O_5$: C, 58.4; H, 6.25%); $[\alpha]_D^{23}$ − 122.7 (*c* 1.10 in acetone) [lit.,[28] $[\alpha]_D^{20}$ − 117.2 (*c* 3.89 in acetone)]; ν_{max}(KCl)/cm^{-1} 3400–3150 br, 1580, 770 and 700; δ_H(250 MHz; D_2O) 3.44 (1 H, dd, *J* 12.4 and 6.2 5'-H), 3.62 (1 H, dd, *J* 12.4 and 3.3, 5-H), 3.92 (1 H, ddd, *J* 6.2, 3.3 and 6.8, 4-H), 4.13 (1 H, dd *J* 4.8 and 1.2, 2-H), 4.19 (1 H, dd, *J* 4.8 and 6.8, 3-H), 5.53 (1 H, d, *J* 1.2, 1-α-H), 6.88–6.97 and 7.17–7.25 (5 H, 2 m).

Cyclohex-3-enyl β-D-*Ribofuranoside* **6**.—The previous procedure was repeated exactly, using cyclohex-3-enyl 2,3,5-tri-*O*-benzoyl-β-D-ribofuranoside (1.0 g, 1.84 mmol) and sodium methoxide solution (4 cm^3). The crude *product* was obtained (0.42 g) and was recrystallised from benzene to give tiny white needles (0.38 g, 90%), m.p. 100–104 °C (Found: C, 57.4; H, 8.0. $C_{11}H_{18}O_5$ requires C, 57.4; H, 7.9%); $[\alpha]_D^{23}$ − 89.9 (*c* 0.74 in acetone); ν_{max}(KCl)/cm^{-1} 3380br, 3020 and 1645w; δ_H(250 MHz; D_2O) 1.39–2.25 (6 H, m), 3.39–3.48 (1 H, 2 dd, *J* 12.2 and 6.5, 5'-H diastereoisomers), 3.60, 3.61 (1 H, 2 dd, *J* 12.2 and 1.7, 5-H diastereoisomers), 3.75–3.87 (3 H, m, 2-, 4- and 1-H), 3.96, 3.97 (1 H, 2 dd, 3-H diastereoisomers), 4.98, 4.99 (1 H, 2 s, 1-α-H diastereoisomers), 5.41 [1 H, d (fine structure), *J* 10.4, C*H*=CH] and 5.53 [1 H, d (fine structure), *J* 10.4, CH=C*H*].

trans-2-*Chlorocyclohexyl* β-D-*Ribofuranoside* **8**.[29]—The previous procedure was repeated exactly, using *trans*-2-chlorocyclohexyl 2,3,5-tri-*O*-benzoyl-β-D-ribofuranoside (1.20 g, 2.07 mmol) and sodium methoxide solution (5 cm^3). The crude product was obtained as a white solid (0.40 g). Recrystallisation from 5% acetone in benzene gave the *title compound* as a 1 : 1 diastereoisomeric mixture (0.34 g, 61%), m.p. 110–138 °C (Found: C, 49.1; H, 7.15; Cl, 13.3. Calc. for $C_{11}H_{19}ClO_5$: C, 49.55; H, 7.2; Cl, 13.3%); $[\alpha]_D^{23}$ − 72.2 (*c* 0.79 in acetone); ν_{max}(mull)/cm^{-1} 3330 br; δ_H(250 MHz; D_2O) 1.00–1.20 (3 H, m), 1.45–1.60 (3 H, m), 1.95–2.15 (2 H, m), 3.41–3.71 (4 H, m, 5-H_2, 1- and 2-H), 3.77–4.03 (3 H, m, 2-, 3- and 4-H), 5.02 and 5.08 (1 H, s, 1-α-H diastereoisomers, 1 : 1). Diastereoisomeric resolution was achieved by recrystallisation of a sample from 1 : 1 acetone–chloroform. The isolated isomer had δ_H(250 MHz; D_2O) 1.02–1.23 (3 H, m), 1.40–1.53 (3 H, m), 1.95–2.05 (2 H, m), 3.42–3.47 (1 H, m, 1- or 2-H), 3.46 (1 H, dd, *J* 6.8 and 12.1, 5'-H), 3.62 (1 H, dd, *J* 3.4 and 12.1, 5-H), 3.60–3.69 (1 H, m, 1- or 2-H), 3.80 (1 H, ddd, *J* 3.4, 6.8 and 6.8, 4-H), 3.92–3.98 (2 H, m, 2- and 3-H) and 5.08 (1 H, s, 1-α-H).

Cyclohex-2-enyl β-D-*Ribofuranoside* **9**.—*trans*-2-Chlorocyclohexyl β-D-ribofuranoside (313 mg, 1.12 mmol) was added to a solution of potassium hydroxide (2.5 g) in methanol (5 cm^3) and the mixture was stirred under reflux for 15 h. After cooling, 50% aqueous methanol (50 cm^3) was added to redissolve the white solids that had been formed. The solution was neutralised with carbon dioxide then evaporated under reduced pressure. The residual solids were digested with acetone and filtered to remove inorganic salts then the filtrate was evaporated. This procedure was repeated, and the resulting pale yellow gum (240 mg) solidified on standing. The *product* was recrystalised from

benzene as waxy plates (116 mg, 43%), m.p. 80–85 °C; $[\alpha]_D^{23}$ −52.3 (*c* 0.39 in methanol); ν_{max}(KCl)/cm^{-1} 3350br, 3020w and 1620w; δ_H(250 MHz; D_2O) 1.37–1.87 (6 H, m), 3.47, 3.48 (1 H, 2 dd, *J* 12.1 and 6.8, 5'-H diastereoisomers), 3.64 (1 H, dd, *J* 12.1 and 3.5, 5-H), 3.79–3.87 (2 H, m, 2-H and 4-H), 3.96–4.03 (1 H, m, 3-H), 4.09–4.16 (1 H, m, 1-H), 5.00, 5.01 (1 H, 2 s, 1-αH diastereoisomers), 5.50–5.63 and 5.79–5.85 (2 H, 2 m, CH=CH).

2-(*Phenoxymethoxy*)*ethyl Acetate* **11a**.—A solution of lithium phenoxide was prepared under nitrogen by adding a solution of phenol (1.88 g, 20.0 mmol) in THF (5 cm^3) to a mixture of 1.57 mol dm^{-3} butyllithium solution in hexane (8.4 cm^3, 13.2 mmol) and THF (5 cm^3) at −70 °C. On occasions, the lithium phenoxide was present as a suspension. To this preparation a solution of 2-(chloromethoxy)ethyl acetate (2.02 g, 13.2 mmol) in THF (5 cm^3) was added dropwise over 5 min. After a further 5 min at −70 °C the mixture was warmed to room temperature and stirred for 1 h. The solution was concentrated under reduced pressure and benzene (30 cm^3) was added. The organic solution was washed with ice-cold 0.1 mol dm^{-3} sodium hydroxide (4 × 25 cm^3) and ice-cold water (2 × 25 cm^3) then dried ($MgSO_4$). Evaporation of solvent under reduced pressure gave a pale yellow oil (2.2 g), which was distilled *in vacuo* to yield the title compound (1.81 g, 65%), b.p. 110–111 °C/0.7 mmHg (Found: C, 62.7; H, 6.8. $C_{11}H_{14}O_4$ requires C, 62.85; H, 6.7%); ν_{max}(film)/cm^{-1} 3050, 3020, 1735, 1595, 760 and 690; δ_H(90 MHz; $CDCl_3$) 2.0 (3 H, s), 3.85 (2 H, t, *J* 5), 4.2 (2 H, t, *J* 5), 5.2 (2 H, s) and 6.9–7.4 (5 H, m).

2-(*Phenoxymethoxy*)*ethanol* **13a**.—A solution of 2-(phenoxymethoxy)ethyl acetate **11a** (2.87 g, 13.7 mmol) in methanol (28 cm^3) was combined with a solution of sodium hydroxide (0.69 g, 16.4 mmol) in water (28 cm^3). The mixture was heated under reflux for 2.5 h, cooled, and diluted with water (28 cm^3). This solution was extracted with ether (3 × 45 cm^3), and the combined extracts washed with ice-cold water (28 cm^3) then dried ($MgSO_4$). Evaporation of solvent under reduced pressure gave an oil (2.0 g) which was distilled *in vacuo* to produce the *title compound* (1.71 g, 74%) b.p. 81–83 °C/0.2 mmHg (Found: C, 64.3; H, 7.3. $C_9H_{12}O_3$ requires C, 64.25; H, 7.2%); ν_{max}(film)/cm^{-1} 3380br, 3050, 3020, 1595, 755 and 690; δ_H(90 MHz; $CDCl_3$) 2.4 (1 H, s, OH), 3.6–3.9 (4 H, m), 5.25 (2 H, s) and 6.85–7.4 (5 H, m); GLC (5% Carbowax; 180 °C) 4.0 min; TLC (silica; ethyl acetate) R_f 0.51.

Reaction of 2-(*Chloromethoxy*)*ethyl Acetate with Lithium Cyclohex-*2-*enyloxide*.—A solution of lithium cyclohex-2-enyloxide was prepared under nitrogen by adding a solution of cyclohex-2-enol (64) (1.96 g, 20.0 mmol) in THF (7.5 cm^3) to a mixture of 1.57 mol dm^{-3} butyllithium solution in hexane (12.75 cm^3, 20.0 mmol) and THF (7.5 cm^3) at −70 °C. After 15 min, a solution of 2-(chloromethoxy)ethyl acetate (3.05 g, 20.0 mmol) in THF (7.5 cm^3) was added dropwise. The mixture was allowed to return to room temperature and stirred for 1 h. Following concentration under reduced pressure, benzene (45 cm^3) was added and the organic solution washed with ice-cold water (3 × 35 cm^3) then brine (35 cm^3) and dried ($MgSO_4$). Evaporation under reduced pressure left a yellow oil (3.3 g), which was distilled *in vacuo* to give *cyclohex-2-enyl acetate* (1.13 g, 40%), b.p. 72–76 °C/15 mmHg (Found: C, 68.7; H, 8.95. Calc. for $C_8H_{12}O_2$: C, 68.55; H, 8.6%); 2-(*cyclohex-2-enyloxymethoxy*)*ethyl acetate* **11b** (0.55 g, 13%, b.p. 72–80 °C/0.01 mmHg).

Reaction of 2-(*Chloromethoxy*)*ethyl Acetate with Cyclohex-*2-*enol and Silver Carbonate, and Subsequent Hydrolysis*.—Cyclohex-2-enol (8.2 g, 83.7 mmol), silver carbonate (30.7 g, 111.3 mmol), and active 4 Å molecular sieves (3 g) were stirred together in dichloromethane (250 cm^3) for 15 min, then 2-(chloromethoxy)ethyl acetate (12.8 g, 83.9 mmol) was added. Carbon dioxide was evolved and a slight rise in temperature to 25–30 °C was noted. The mixture was protected from moisture and stirred in the dark for 48 h at ambient temperature. Filtration and evaporation of solvent under reduced pressure left an oil (19.8 g) which was distilled *in vacuo*. Volatile material was discarded and the fraction b.p. 75–80 °C/0.03 mmHg or b.p. 85–90 °C/0.1 mmHg was collected (6.8 g). This sample was dissolved in methanol (60 cm^3) and the solution added to sodium hydroxide (1.26 g, 31.5 mmol) dissolved in water (60 cm^3). Organic materials were extracted with ether (3 × 100 cm^3) and the combined extracts washed with brine (100 cm^3) then dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave a pale oil (5.53 g). Chromatography on silica gel using 5% ethyl acetate in chloroform followed by 50% ethyl acetate in chloroform yielded *bis*(*cyclohex-2-enloxy*)*methane* **12** as a colourless oil (1.29 g, 15%) (Found: C, 74.2; H, 9.7. $C_{13}H_{20}O_2$ requires C, 74.95; H, 9.7%); ν_{max}(film)/cm^{-1} 3020 and 1650; δ_H(250 MHz; $CDCl_3$) 1.53–2.15 (12 H, m), 4.19 (2 H, m), 4.83 (2 H, s), 5.77 (2 H, m) and 5.88 (2 H, m); GLC (5% apiezon; 200 °C) 5.2 min; and 2-(*cyclohex-2-enyloxymethoxy*)*ethanol* **13b** as a colourless oil (3.58 g, 25%) (Found: C, 62.8; H, 9.4. $C_9H_{16}O_3$ requires C, 62.75; H, 9.35%); ν_{max}(film)/cm^{-1} 3420 br, 3020 and 1650; δ_H(250 MHz; $CDCl_3$) 1.54–2.07 (6 H, m), 2.69 (1 H, s, OH), 3.74 (4 H, m), 4.14 (1 H, m), 4.81 (2 H, s), 5.77 (1 H, m) and 5.90 (1 H, m); GLC (5% Apiezon; 220 °C) 2.1 min.

Reaction of 2-(*Chloromethoxy*)*ethyl Acetate with Cyclohex-*3-*enol and Silver Carbonate, and Subsequent Hydrolysis*.—The previous procedure was repeated using cyclohex-3-enol (10.0 g, 102 mmol), silver carbonate (37.4 g, 136 mmol) and 2-(chloromethoxy)ethyl acetate (15.5 g, 102.6 mmol). Distillation under reduced pressure gave a fraction b.p. 75–80 °C/0.05 mmHg or b.p. 90–95 °C/0.2 mmHg as a clear oil (9.5 g). After hydrolysis, another oil was obtained (8.2 g). Chromatography on silica gel using 10% ethyl acetate in chloroform followed by 50% ethyl acetate in chloroform yielded *bis*(*cyclohex-*3-*enyloxy*)*methane* **12c** as a colourless oil (2.98 g, 28%) (Found: C, 73.9; H, 9.85. $C_{13}H_{20}O_2$ requires C, 74.95; H, 9.7%); ν_{max}(film)/cm^{-1} 3020 and 1645; δ_H(250 MHz; $CDCl_3$) 1.25–2.44 (12 H, m), 3.92 (2 H, m), 4.85 (2 H, s), and 5.62 (4 H, m); GLC (5% Apiezon; 200 °C) 4.9 min; and 2-(*cyclohex-*3-*enyloxymethoxy*)*ethanol* **13c** as a colourless oil (4.58 g, 26%) (Found: C, 62.8; H, 9.5. $C_9H_{16}O_3$ requires C, 62.75; H, 9.35%); ν_{max}(film)/cm^{-1} 3430br, 3020 and 1645; δ_H(250 MHz; $CDCl_3$) 1.59–2.43 (6 H, m), 2.73 (1 H, s, OH), 3.73 (4 H, m), 3.89 (1 H, m), 4.82 (2 H, s) and 5.62 (2 H, m); GLC (5% Apiezon; 200 °C) 2.1 min.

Reaction of 2-(*Chloromethoxy*)*ethyl Acetate with Cyclohexanol and Silver Carbonate*.—Cyclohexanol (2.25 g, 22.5 mmol), silver carbonate (8.0 g, 29 mmol) and active 4 Å molecular sieves (2 g) were stirred together in dichloromethane (65 cm^3) for 15 min, then 2-(chloromethoxy)ethyl acetate (3.43 g, 22.05 mmol) was added. Following effervescence and slight warming the mixture was stirred in the dark for 48 h whilst protected from moisture. The mixture was then filtered and evaporated under reduced pressure to leave an oil (3.8 g). Distillation under reduced pressure gave two fractions. The first fraction (0.8 g) b.p. 40–60 °C/0.1 mmHg contained a component which co-eluted with ethylene glycol monoacetate when analysed by GLC. The second fraction (2.7 g) b.p. 85–90 °C/0.1 mmHg was subjected to chromatography on silica gel using 5% ethyl acetate in chloroform followed by 10% ethyl acetate in chloroform, and yielded *bis*(*cyclohexyloxy*)*methane* as a colourless oil (0.60 g, 25%) (Found: C, 73.85; H, 11.6. Calc. for

$C_{13}H_{24}O_2$: C, 73.55; H, 11.4%); δ_H(250 MHz; $CDCl_3$) 1.14–1.94 (20 H, m), 3.57 (2 H, tt, *J* 9.4 and 4.0) and 4.78 (2 H, s); GLC (5% Apiezon; 180 °C) 12.5 min; and 2-(*cyclohexyloxymethoxy*)*ethyl acetate* as a colourless oil (1.36 g, 28%) (Found: C, 61.4; H, 9.4. $C_{11}H_{20}O_4$ requires C, 61.1; H, 9.3%); ν_{max}(film)/cm^{-1} 1735; δ_H(250 MHz; $CDCl_3$) 1.17–1.93 (10 H, m), 2.09 (3 H, s), 3.53 (1 H, m), 3.76 (2 H, t, *J* 4.7), 4.24 (2 H, t, *J* 4.7) and 4.77 (2 H, s); GLC (5% Apiezon; 180 °C) 7.3 min.

Reaction of 2-(*Chloromethoxy*)*ethyl Acetate with Benzyl Alcohol and Silver Carbonate, and Subsequent Hydrolysis*.—The previous procedure was repeated using benzyl alcohol (2.43 g, 22.5 mmol) instead of cyclohexanol. The reaction was subjected to work-up after 24 h, and distillation of the oil (5.1 g) under reduced pressure gave two fractions. The first fraction (1.2 g) b.p. 45–65 °C/0.2 mmHg contained a component which co-eluted with ethylene glycol monoacetate (87) when analysed by GLC. The second fraction (3.0 g) b.p. 105–110 °C/0.2 mmHg was dissolved in methanol (30 cm^3) and this solution added to sodium hydroxide (1.0 g, 25 mmol) in water (30 cm^3). The mixture was heated under reflux for 2 h, cooled, diluted with water (30 cm^3), and extracted with ether (3 × 50 cm^3). Combined extracts were washed with brine (50 cm^3) and dried ($MgSO_4$). Evaporation of solvent under reduced pressure gave an oil (2.2 g). Flash chromatography using chloroform as eluent yielded *bis*(*benzyloxy*)*methane* as a colourless oil (0.39 g, 15%) (Found: C, 78.7; H, 6.9. $C_{15}H_{16}O_2$ requires C, 78.9; H, 7.05%); ν_{max}(film)/cm^{-1} 3080, 3060, 3025, 735 and 700; δ_H(250 MHz; $CDCl_3$) 4.66 (4 H, s), 4.85 (2 H, s) and 7.30–7.37 (10 H, m); GLC (5% Apiezon; 200 °C) 15.6 min; and 2-(*benzyloxymethoxy*)-*ethanol* as a colourless oil (1.76 g, 43%) (Found: C, 65.6; H, 7.8. $C_{10}H_{14}O_3$ requires C, 65.9; H, 7.75%): ν_{max}(film)/cm^{-1} 3410br, 3060, 3025, 1600w, 1580w, 750 and 700; δ_H(250 MHz; $CDCl_3$) 2.44 (1 H, br s, OH), 3.74 (4 H, m), 4.64 (2 H, s), 4.81 (2 H, s) and 7.28–7.38 (5 H, m); GLC (5% Apiezon; 220 °C) 4.0 min.

Sodium 2-(*Phenoxymethoxy*)*ethyl Sulfate* **14a**.—Freshly prepared pyridine–sulfur trioxide complex (575 mg, 4.76 mmol) was added to a solution of 2-(phenoxymethoxy)ethanol **13a** (800 mg, 4.76 mmol) in pyridine (10 cm^3) and the mixture was stirred at room temperature for 24 h with the exclusion of moisture. The resulting pale yellow solution was evaporated under reduced pressure, and the residue co-evaporated with pyridine (2 × 20 cm^3), finally at 0.1 mmHg, to leave a yellow syrup (1.63 g). This material was dissolved in water (50 cm^3) and passed through a column of ion-exchange resin in the Na^+-form. The eluate was freeze-dried to give the product as a powdery white solid (1.10 g, 85%), m.p. 145–147 °C (decomp.); λ_{max}(pH 9 10 mmol dm^{-3} phosphate buffer)/nm 262sh, 267 and 273 (ε_{267} 950 mol^{-1} dm^{-3} cm^{-1}); ν_{max}(KCl)/cm^{-1} 3570, 3440, 3050w, 3020w, 1630, 1595, 1585, 780, 755 and 690; δ_H(250 MHz; D_2O) 3.78 [2 H, t (fine structure)], 3.98 [2 H, t (fine structure)], 5.14 (2 H, s), 6.90–6.95 and 7.16–7.23 (5 H, 2 m); HPLC (275 nm; 50:50 MeOH–pH 9 buffer) 4.2 min (100%). Samples were routinely dissolved in pH 9.0 10 mmol dm^{-3} phosphate buffer immediately after preparation, to prevent the otherwise rapid hydrolysis of the acetal function to liberate phenol. Samples containing phenol [HPLC (275 nm; 50:50 MeOH–pH 9 buffer) 11.4 min] could be purified by medium pressure chromatography on reversed phase ODS-silica using methanol–water (40:60) as eluent.

Sodium 2-(*Cyclohex-2-enyloxymethoxy*)*ethyl Sulfate* **14b**.—The previous procedure was repeated exactly, using pyridine-sulfur trioxide complex (600 mg, 3.77 mmol), 2-(cyclohex-2-enyloxymethoxy)ethanol **13b** (650 mg, 3.78 mmol) and pyridine (7.5 cm^3). The product was obtained as a powdery white solid (1.00 g, 97%), m.p. 74–77 °C (decomp.); ν_{max}(KCl)/cm^{-1} 3580, 3460, 3020 and 1630; δ_H(90 MHz; D_2O) 1.5–2.1 (6 H, m), 3.8–3.95 (2 H, m), 4.1–4.3 (3 H, m), 4.85 (2 H, s) and 5.7–6.1 (2 H, m).

Sodium 2-(*Cyclohex-3-enyloxymethoxy*)*ethyl Sulfate* **14c**.—The previous procedure was repeated exactly, using pyridine-sulfur trioxide complex (610 mg, 3.84 mmol) and 2-(cyclohex-3-enyloxymethoxy)ethanol **13c** (660 mg, 3.84 mmol). The product was obtained as a powdery white solid (1.00 g, 95%), m.p. 64–67 °C (decomp.); ν_{max}(KCl)/cm^{-1} 3540, 3020 and 1630; δ_H(90 MHz; D_2O) 1.5–2.3 (6 H, m), 3.8–3.9 (2 H, m), 3.9–4.1 (1 H, br m), 4.1–4.2 (2 H, m), 4.85 (2 H, s) and 5.5–5.8 (2 H, m).

3-*Acetyl*-N-*ethylpyridinium Bromide*.—3-Acetylpyridine (24.2 g, 0.02 mol) and ethylbromide (21.8 g, 0.2 mol) in dry acetonitrile (50 cm^3) were refluxed overnight. The solvent was then removed under reduced pressure and gave the required compound (38.2 g, 83%) m.p. 100 °C (Found: C, 46.5; H, 5.2; Br, 34.3; N, 6.0. $C_9H_{12}BrNO$ requires C, 46.87; H, 5.25; Br, 34.68; N, 6.08%); δ_H(90 MHz; D_2O) 1.75 (t, CH_3), 2.9 (s, CH_3), 5.22 (m, CH_2) and 8.3–10.22 (m, 4 H, pyridine).

Similarly prepared was 3-*acetyl*-N-*benzylpyridinium bromide* (89%), m.p. 171–174 °C (Found: C, 57.6; H, 4.8; Br 27.2; N, 4.8. $C_{14}H_{14}BrNO$ requires: C, 57.55; H, 4.83; Br, 27.35; N, 4.79%); δ_H(90 MHz; D_2O) 2.9 (s, CH_3), 6.5 (s, CH_2), 7.35 (m, C_6H_5) and 8.2–10.6 (m, 4 H, pyridine).

3-α-*Bromoacetyl*-N-*ethylpyridinium Bromide* **15**.—3-Acetyl-*N*-ethylpyridinium bromide (0.65 g, 2.84 × 10^{-3} mol) was dissolved in chloroform–ether (10 cm^3/1 cm^3) and the solution cooled in an ice-salt bath. Bromine (0.45 g, 2.84 × 10^{-3} mol) was added dropwise with vigorous stirring and under cooling for 1 h and for 0.5 h at room temperature. The solvent was removed under reduced pressure to give an orange oil which was recrystallised from ethanol to give the required compound bromoacetyl-*N*-ethylpyridinium bromide **15** (0.84 g, 96%), m.p. 105–107 °C (Found: C, 34.1; H, 3.4; Br, 51.04; N, 4.4. $C_9H_{11}Br_2NO$ requires: C, 33.90; H, 3.55; Br, 51.55; N, 4.51%); δ_H(90 MHz; D_2O) 1.60 (m, CH_3), 3.85 (s, CH_2Br), 4.75 (m, CH_2) and 8.1–9.4 (m, 4 H, pyridine).

Similarly prepared was 3α-*bromoacetyl*-N-*benzylpyridinium bromide* **16** (90.5%) m.p. 175–177 °C (Found: C, 45.6; H, 3.5; Br, 44.2; N, 3.9. $C_{14}H_{13}Br_2NO$ requires C, 45.28; H, 3.50; Br, 43.13; N, 3.77%): δ_H(90 MHz; D_2O) 3.3 (s, CH_2Br), 5.9 (m, CH_2), 7.5 (s, C_6H_5) and 8.1–9.5 (m, pyridine).

N-*Methylthioformamide*.—Phosphorus pentasulfide (6 g, 0.026 mol) was suspended in dry dioxane and *N*-methylformamide (6 g, 0.1 mol) rapidly added. The reaction mixture was then brought to reflux for 1 h, allowed to cool and the dioxane solution decanted off. The clear dioxane solution was evaporated under reduced pressure to give the required compound as a light orange liquid (4.55 g, 61%) (Found: C, 32.0; H, 6.75; N, 18.5; S, 41.8. C_2H_5NS requires C, 31.97; H, 6.71; N, 18.64; S, 42.68%); δ_H(90 MHz; DMSO) 3.1 (d, CH_3), 9.25 (s, CSH) and 10.2 (s, NH).

Similarly prepared was N-*benzylthioformamide* (70%) (Found: C, 63.7; H, 5.9; N, 9.1; S, 20.8. C_8H_9NS requires C, 63.53; H, 6.0; N, 9.26; S, 21.20%); δ_H(90 MHz; DMSO) 4.75 (d, CH_2), 7.40 (s, C_6H_5), 9.45 (s, CSH) and 10.6 (s, NH).

2-*Bromomethyl*-N-*methylthiazolium Bromide* **17**.—A cooled acetone solution of 1,3-dibromoacetone (4.32 g, 0.02 mol in 10 cm^3 cold acetone) was added with stirring to a solution of *N*-methylthioformamide (1.5 g, 0.02 mol in 15 cm^3 cold acetone). Stirring was continued for 3 days at room temperature. The acetone was evaporated under reduced pressure. Ether was added and the insoluble products were removed by filtration. The residue was crystallised from methanol–ethyl acetate

(40/60) mixture and gave a dark brown solid of 2-bromomethyl-*N*-methylthiazolium bromide (2.14 g, 39%); m.p. 164–166 °C (decomp.) (Found: C, 22.0; H, 2.4; Br, 58.3; N, 5.1; S, 12.2. $C_4H_7Br_2NS$ requires C, 22.01; H, 2.56; Br, 58.6; N, 5.13; S, 11.73); δ_H(90 MHz; DMSO) 4.30 (s, NCH_3), 5.15 (s, $BrCH_2$), 8.52 (d, CH) and 10.32 (d, H).

Similarly prepared was *2-bromomethyl-N-benzylthiazolium bromide* **18** (53.6%) m.p. 18 °C (decomp.) (Found: C, 38.4; H, 3.1; Br, 43.2; N, 3.9; S, 10.3. $C_{10}H_{11}Br_2NS$ requires C, 37.86; H, 3.18; Br, 45.80; N, 4.01; S, 9.17%); δ_H(90 MHz; DMSO), 5.10 (s, CH_2), 5.95 (s, $BrCH_2$), 7.50 (s, C_6H_5), 8.65 (d, CH) and 10.28 (d, CH).

Enzyme Modification

Carboxypeptidase A.—Polythene storage bottles, reaction vessels, and pipette tips were soaked for 24 h in two changes of 30% nitric acid then rinsed thoroughly with doubly distilled water. Nylon clips for sealing dialysis tubing were boiled for 2 h in alkaline 0.1 mol dm^{-3} EDTA solution. Dialysis tubing was soaked in two changes of saline buffer containing EDTA. Water was doubly distilled from an all-glass apparatus. Saline buffers were prepared using analytical grade sodium chloride (Proalys A. R., May and Baker). Buffer aliquots (500 cm^3) were routinely washed with 0.01% w/v dithizone solution[41] in distilled carbon tetrachloride (typically 5 × 100 cm^3), and were stored in clean polythene bottles. Metal salts were used as follows: cobalt(II) chloride hexahydrate and nickel(II) chloride hexahydrate, both Specpure grade (Johnson Matthey Chemicals); rhodium(III) chloride, ruthenium(III) chloride and palladium(II) chloride, all reagent grade (Lancaster Synthesis). Bovine pancreatic carboxypeptidase A_α was obtained commercially as an aqueous suspension (Sigma Chemical Co., product no. C 0261, lot 24F-8080). 1,10-Phenanthroline was used as commercially supplied (Sigma Chemical Co.).

Solution CPA protein concentrations were established by UV spectroscopy using a Pye Unicam SP 800 Å Ultraviolet Spectrophotometer, and were calculated on the basis of a literature[42] molar absorptivity value of 6.42×10^4 mol^{-1} dm^3 cm^{-1} at 278 nm. UV–VIS electronic absorption spectra of CPA samples were recorded on a Shimdazu UV-250 Recording Spectrophotometer interfaced to a Hewlett Packard 86B Microcomputer. Data was manipulated using software written by Dr. I. R. Dunkin, Department of Pure and Applied Chemistry, University of Strathclyde. Standard solutions for metal analysis calibration curves were prepared from metal chlorides in 1 mol dm^{-3} sodium chloride solution.

Apo-carboxypeptidase A. All apo-CPA samples were prepared in the following manner. From an aqueous suspension of CPA (*ca.* 23 mg cm^{-3}) a sample was removed (3 cm^3; *ca.* 69 mg, 2×10^{-6} mol) and was centrifuged at 9000 rpm and 0 °C for 15 min. The supernatant was discarded and the solids washed with ice-cold water then recentrifuged. A 10^{-3} mol dm^{-3} solution was prepared by dissolving the protein solids in pH 7.5 1 mol dm^{-3} sodium chloride/0.1 mol dm^{-3} Tris buffer (2 cm^3) at 0 °C. After 12 h the solution was centrifuged and the supernatant transferred to a dialysis bag which was then sealed. This solution was dialysed at 0 °C in a polythene vessel against pH 7.5 1 mol dm^{-3} sodium chloride/0.1 mol dm^{-3} Tris buffer containing 1.0 mmol dm^{-3} 1,10-phenanthroline (4 × 100 cm^3) then against pH 7.5 1 mol dm^{-3} sodium chloride/0.1 mol dm^{-3} Tris buffer (4 × 100 cm^3). Each dialysis lasted 12 h. A sample of apo-CPA prepared in this manner was assayed for peptidase activity and was found to have 2.2% of the native enzyme activity.

Metal-substituted carboxypeptidase A: cobalt, nickel, rhodium (method 1*), ruthenium and palladium.* An apo-CPA sample was dialysed at 0 °C in a polythene vessel against 1.0 mmol dm^{-3} metal chloride solution in pH 7.5 1 mol dm^{-3} sodium chloride/5 mmol dm^{-3} Tris buffer (2 × 100 cm^3) then against 0.2 mmol dm^{-3} metal chloride solution in pH 7.5 2.5 mmol dm^{-3} sodium chloride/0.3 mmol dm^{-3} Tris buffer (100 ml). Each dialysis lasted 12 h. Solids were collected by centrifugation at 9000 rpm and 0 °C for 30 min, and were washed with ice-cold water then recentrifuged. The resulting solids were stirred in pH 7.5 1 mol dm^{-3} sodium chloride/5 mmol dm^{-3} Tris buffer (3 cm^3) at 0 °C for 12–24 h to redissolve soluble protein, and the solution was separated from insoluble material by centrifugation. The supernatant was collected and stored at 0 °C.

Metal-substituted carboxypeptidase A: rhodium (method 2*).* An apo-CPA sample was dialysed at 0 °C in a polythene vessel against 1.0 mmol dm^{-3} rhodium chloride solution at pH 7.5 1 mol dm^{-3} sodium chloride/5 mmol dm^{-3} Tris buffer (2 × 100 cm^3) then against the metal-free buffer (3 × 100 cm^3). Each dialysis lasted 12 h. Solids were removed by centrifugation at 9000 rpm and 0 °C for 15 min. The supernatant was collected and stored at 0 °C. Protein concentration was found to be 8.10×10^{-5} mol dm^{-3} while ICPES metal analysis showed the rhodium concentration to be only $1.20 \times 10^{-5} \pm 0.36 \times 10^{-5}$ mol dm^{-3}.

3α-Acetyl-N-ethylpyridinium-modified papain **19**. Papain (200 mg, 8.6×10^{-6} mol) was dissolved in pH 7 phosphate buffer (4 cm^3 0.02 mol dm^{-3}) and was mixed with a five fold excess of 3-α-bromoacetyl-*N*-ethylpyridinium bromide **15** (13.47 mg, 4.3×10^{-5} mol). The mixture was stirred for 5 h in an ice bath. Then the second five-fold molar excess of pyridinium salt **15** was added to the solution with a five-fold molar excess of cysteine (7.55 mg, 4.3×10^{-5} mol) and stirred overnight in an ice bath. The third five-fold excess of pyridinium salt **15** with a five-fold excess of cysteine was added and stirring continued for another 5 h in an ice bath. The modified-papain was separated from excess pyridinium salt and cysteine by dialysis against double distilled water at 4 °C for 24 h. Freeze drying gave a crude powder of modified papain **19** (150 mg, 75%).

Similarly prepared were *3α-acetyl-N-benzylpyridinium-modified papain* **20** in 75% yield from **16**, *N-methylthiazolium-modified papain* **21** in 70% yield from **17** and *N-benzylthiazolium-modified papain* **22** in 70% yield from **18**.

Purification of Modified Papains.—All purification procedures were carried out at 4 °C.

1. *Dialysis*. The procedure was the same final step as that described for the modification of papain above and was carried out for 24–49 h.

2. *Gel filtration*. Partially purified modified papain (50 mg) was dissolved in double distilled water (3 cm^3) and applied to a column of Sephadex G50 (20–35 × 1.5 cm) eluting with double distilled water. Fractions containing protein were identified by UV absorption and Bradfoed's assay for protein.[43]

3. *Absorption of unchanged papain*. Partially purified modified papain (50 mg) was dissolved in double distilled water and activated thiol Sepharose (70 mg)[44] added. The solution was stirred for 20 h after which time the Sepharose was removed by centrifugation (10 min, 200 rpm) and filtration through sintered glass.

The hydrolytic activity of papain was followed through each purification step by standard spectrophotometric methods using *N*-benzoylarginine ethyl ester (at pH 6.5) or *N*-benzoylarginine 4-nitroanilide (at pH 7.5).

Reactions of Modified Enzymes

Hydrogenase Studies with Metal-substituted CPA.—Metal-substituted CPA samples were used within one week of preparation. Stock solutions of *N*-acyl dehydrophenylalanines **1–4** were prepared in 0.1 mol dm^{-3} sodium hydroxide solution immediately before use. Glass reaction flasks were soaked in a

Table 3 Reaction mixtures for hydrogenation experiments

Entry	Metallo-CPA	Substrate	Enzyme Concentration (mol dm^{-3})	Substrate Concentration (mol dm^{-3})
(a)	Zn-CPA[a]	**1**	6×10^{-5}	2.5×10^{-3}
(b)	Zn-CPA[a]	**2**	6×10^{-5}	2.5×10^{-3}
(c)	Co-CPA	**1**	3×10^{-5}	3.0×10^{-4}
(d)	Co-CPA	**2**	3×10^{-5}	3.0×10^{-4}
(e)	Co-CPA	**3**	3×10^{-5}	3.0×10^{-4}
(f)	Co-CPA	**4**	3×10^{-5}	3.0×10^{-4}
(g)	Ni-CPA	**1**	5×10^{-5}	3.0×10^{-4}
(h)	Ni-CPA	**2**	5×10^{-5}	3.0×10^{-4}
(i)	Ni-CPA	**3**	5×10^{-5}	3.0×10^{-4}
(j)	Ni-CPA	**4**	5×10^{-5}	3.0×10^{-4}
(k)	Rh-CPA[b]	**1**	5×10^{-5}	5.0×10^{-4}
(l)	Rh-CPA[b]	**2**	5×10^{-5}	5.0×10^{-4}
(m)	Rh-CPA[b]	**3**	5×10^{-5}	5.0×10^{-4}
(n)	Rh-CPA[b]	**4**	5×10^{-5}	5.0×10^{-4}
(o)	none[c]	**2**	0	5.0×10^{-4}

[a] Native enzyme. [b] Prepared by method 2. [c] Control experiment carried out in the presence of 10^{-3} mol dm^{-3} rhodium(III), but without the presence of CPA in any form.

Table 4 Analytical HPLC systems for hydrogenation reactions

Mobile phase	Detector Wavelength/nm	Component	Retention time (min)
30:70		**1**	9.7
MeOH–pH 9.0	225	**2**	6.7
10 mmol dm^{-3}			
phosphate buffer		benzoyl-Phe	11.6
5:95		**3**	8.0
MeOH–pH 9.0	215	**4**	6.0
10 mmol dm^{-3}			
phosphate buffer		acetyl-Phe	10.3

mixture of concentrated sulfuric and nitric acids (1:1) for several days then rinsed with doubly distilled water.

Hydrogenation Experiments.—Each of the mixtures shown as entries (a)–(o) in Table 3 was prepared in pH 7.5 1 mol dm^{-3} sodium chloride 5 mmol dm^{-3} Tris buffer to give a total reaction volume of 2 cm^3 and was incubated under an atmosphere of hydrogen gas (1 atmos.) at ambient temperature for 24 h. Samples were removed and diluted then analysed by the appropriate HPLC system.

Results indicated that the mixtures shown as entries (a)–(k) and (m) contained only the starting material component. Entries (l), (n) and (o) contained the starting material component plus a small amount of the *cis*/*trans*-isomerisation product component. In each of the three cases, conversion was estimated at *ca.* 5–10%, on the basis of peak integrations, and assuming identical ε values for (*Z*)- and (*E*)-isomers at the chosen analytical wavelength.

Analysis Procedures.—HPLC analyses were performed using a Waters ODS-2 reversed phase column and a Cecil Instruments CE 212 Variable Wavelength Ultraviolet Monitor at a constant eluent flowrate of 42 cm^3 h^{-1}. Separations of **1**, **2** and benzoylphenylalanine and of **3**, **4** and acetylphenylalinine were achieved and detected using conditions shown in Table 4.

Oxygen Transferase Studies with HLADH.—Homogeneous solutions of anisole and cyclohexene (typically 12 and 2 mmol dm^{-3} respectively) were obtained by shaking an excess of the organic compound (500 mm^3) vigorously with pH 9.0 10 mmol dm^{-3} phosphate buffer (50 cm^3) for 1 h, subjecting the mixture

Table 5 Reaction mixtures for oxygen transfer experiments[a]

Entry	Substrate/Oxidant	Reaction Conditions[b]	Reaction time (h)	Work-up and Analysis Procedure[c]
(a)	anisole–H_2O_2	d	18	A
(b)	anisole–TBHP	d	18	A
(c)	cyclohexene–H_2O_2	d,e	22	B
(d)	cyclohexene–TBHP	d,f	29	B
(e)	**5**–H_2O_2	g	18	C
(f)	**5**–TBHP	g	18	C
(g)	**8**–H_2O_2	g	20	D
(h)	**8**–TBHP	g	20	D
(i)	**6**–H_2O_2	g	18	D
(j)	**6**–TBHP	g	18	D
(k)	**14a**–H_2O_2	standard	18	C
(l)	**14a**–TBHP	standard	18	C
(m)	**14b**–H_2O_2	standard	40	D
(n)	**14b**–TBHP	standard	40	D
(o)	**14c**–H_2O_2	standard	24	D
(p)	**14c**–TBHP	standard	24	D

[a] Reactions carried out in two parallel experiments; in one partner NAD was added (a)–(d), or AMP (e)–(p). [b] Standard refers to conditions in the text; footnotes d–g give modifications. [c] See text. [d] [Enzyme] = 1.0×10^{-5} mol dm^{-3}. [e] [substrate] = 1.25×10^{-3} mol dm^{-3}, [oxidant] = 1.6×10^{-3} mol dm^{-3}. [f] [substrate] = [oxidant] = 1.9×10^{-3} mol dm^{-3}. [g] pH 7.0 10 mmol dm^{-3} phosphate buffer.

to centrifugation at 1000 rpm for 10 min, then removing the aqueous phase carefully by pipette. Anisole concentration was established by UV spectroscopy based on a molar absorptivity value of 1480 mol^{-1} dm^3 cm^{-1} at 260 nm in aqueous solution.[45] Cyclohexene concentration was established by GLC (10% Carbowax; 110 °C) using solutions of cyclohexene in ethanol to construct calibration curves.

Hydrogen peroxide (Fisons) and *tert*-butyl hydroperoxide (Aldrich Chemical Co.) were obtained commercially and diluted with doubly distilled water. Concentrations were established by titrimetric analysis.[46]

Alcohol dehydrogenase from equine liver was obtained commercially as a lyophilised solid (Sigma Chemical Co., product no. A 6128, lots 81F-8040 and 124F-9022). NAD$^+$ and AMP were used as commercially supplied (Sigma Chemical Co.). Solutions of modified acyclic NAD fragments in pH 9.0 10 mmol dm^{-3} phosphate buffer were used within three days of preparation.

Oxygen Transfer Experiments.—Each of the substrate and oxidant combinations shown as entries (a)–(p) in Table 5 were incubated together with HLADH in the dark and at ambient temperature under the following standard conditions, unless otherwise stated: oxidant 10^{-2} mol dm^{-3}, substrate 10^{-2} mol dm^{-3}, HLADH 1.25×10^{-5} mol dm^{-3}, in pH 9.0 10 mmol dm^{-3} phosphate buffer (10 cm^3). Experiments were performed in duplicate, and on the second occasion coenzyme (NAD$^+$) or coenzyme fragment (AMP) was added at a concentration of 10^{-3} mol dm^{-3}. After the reaction times noted in Table 5, mixtures were subjected to the appropriate work-up and analysis procedures.

In experiments both with and without the presence of coenzyme or coenzyme fragment, the following observations were made. Entries (a) and (b) produced none of the three methoxyphenols, while entries (c) and (d) contained cyclohexene but no cyclohexene oxide or *trans*-cyclohexane-1,2-diol. Entries (e), (f), (k) and (l) yielded phenol as the only hydroxylated aromatic product. Cyclohex-2-enol was generated upon work-up of entries (g), (h), (m) and (n), as was cyclohex-3-enol from entries (i), (j), (o) and (p), but these materials were rarely detected in the final NMR spectroscopic analysis due to their affinity for the ion-exchange resin used in the work-up

procedures. NMR spectra of work-up products from entries (g)–(j) showed the presence of D-ribose, but none of the samples from entries (e)–(j) and (m)–(p) contained a cyclohexanetriol.

Work-up and Analysis Procedures.—Procedure A. After the reaction time, ammonium sulfate (0.5 g) was added and the incubation mixture was shaken to achieve dissolution. The solution was acidified with 2 mol dm^{-3} hydrochloric acid (1 cm^3) and was extracted with ether (4 × 5 cm^3). Extracts were combined, filtered, and dried ($MgSO_4$), then evaporated under reduced pressure. The residue was redissolved in ethyl acetate (1.0 cm^3) and the resulting solution analysed by GLC (5% FFAP; 210 °C). The following retention times were relevant:

ethyl acetate (solvent)	0.44–1.5 min
anisole	< 1.5 min (eluted with solvent)
tert-butanol (TBHP by-product)	< 1.5 min (eluted with solvent)
o-methoxyphenol	2.0 min
m-methoxyphenol	9.3 min
p-methoxyphenol	7.5 min

Procedure B. After the reaction time, ammonium sulfate (0.5 g) was added and the incubation mixture was shaken to achieve dissolution. Organic materials were extracted with ethyl acetate (4 × 5 cm^3) and the combined extracts were dried ($MgSO_4$). Following concentration by distillation at atmospheric pressure, the liquid residue (*ca.* 1 cm^3) was analysed by GLC (a: 10% Carbowax; 110 °C. b: 5% Carbowax; 150 °C). The following retention times were relevant:

a:	ethylacetate (solvent)	0.4–0.9 min (1.7 min, impurity)
	cyclohexene	1.0 min
	cyclohexene oxide	2.8 min
b:	ethyl acetate (solvent)	0.3–1.2 min
	cyclohexene	< 1.2 min (eluted with solvent)
	trans-cyclohexane-1,2-diol	5.6 min

Procedure C. After the reaction time, the incubation mixture was acidified to pH 2.0 with concentrated hydrochloric acid, and boiled on a steam bath for 30 min, then chilled in ice for 5 min. The resulting solution was sampled and diluted, and analysed by HPLC using a Waters ODS-2 reversed phase column and a Cecil Instruments CE 212 Variable Wavelength Ultraviolet Monitor. With methanol: pH 3.0 50 mmol dm^{-3} phosphate buffer (45:55) as the mobile phase, at a constant flow rate of 42 cm^3 h^{-1}, and an optimum detection wavelength of 280 nm, the following retention times were relevant:

phenol	12.0 min
catechol	7.0 min
resorcinol	5.3 min
quinol	4.3 min

Procedure D. After the reaction time, the incubation mixture was acidified to pH 2.0 with concentrated hydrochloric acid, and boiled on a steam bath for 30 min, then chilled in ice for 5 min. The mixture was then passed through a column of mixed-bed ion-exchange resin (5 g dry) which was then washed with water (10 cm^3). The eluent was evaporated under reduced pressure and the residue dissolved in deuterium oxide. The resulting solution was examined by 250 MHz proton NMR spectroscopy.

Reactions of Pyruvate with Thiazolium Salt Modified Papains.—To a solution of sodium phosphate buffer (2 cm^3, pH7, 0.5 mol dm^{-3}) in a UV cell a solution of NADH (0.1 cm^3, 4.7 mmol dm^{-3}) and sodium pyruvate (0.1 cm^3, 2 mmol dm^{-3}) in the same buffer were added and mixed. A solution of horse liver alcohol dehydrogenase (20 mm^3, 10 μmol dm^{-3}) in buffer was added and the change in UV absorption at 340 nm observed; a small decrease was apparent. Further controls were carried out by adding acetaldehyde to the above solution giving a rapid decrease in absorbance, and papain which caused only a small decrease. The effect of the modified papain **21** was observed by incubating a solution of this protein (100 mm^3, 20 μmol dm^{-3}) with sodium pyruvate solution (200 mm^3) for 5 min. Buffer solution (1.9 cm^3) and NADH solution (0.1 cm^3) were added and the assay reaction initiated by alcohol dehydrogenase solution (20 mm^3). The absorption at 340 nm decreased rapidly to zero within 2–3 min indicating the presence of acetaldehyde. A complementary series of experiments was carried out measuring the loss of pyruvate with lactate dehydrogenase under the same concentration conditions. In this case the control experiments showed a rapid decrease in the absorption at 340 nm and no significant absorption decrease after the pyruvate samples had been pre-incubated with modified papain **21**.

Reduction of Electrophilic Substrates by Pyridinium-modified Papains **19**, **20**.—Pyridinium-modified papain **19** or **20** (10 mg, 4 × 10^{-7} mol) was dissolved in pH 7.0 phosphate buffer (10 cm^3) and stirred for 0.5 h at room temperature. Sodium dithionite (150 mg, 8.6 × 10^{-4} mol) was added into the solution and stirred for 1 h. The solution became a little turbid and UV spectroscopy showed the presence of 1,4-dihydropyridine. Ethyl pyruvate (100 mg, 9.79 × 10^{-7} mol) was added to the solution which was incubated for two days. After this time, UV spectroscopy showed that the dihydropyridine was no longer present. The reaction solution was extracted with ether (3 × 10 cm^3) and the products analysed by GLC (column, FFAP) in comparison with a standard solution.

Reduction of α,α,α-Trifluoroacetophenone Using 4-Dihydronicotinamides as Recycling Agents.—Pyridinium papain **19** or **20** (10 mg, 4 × 10^{-7} mol) was dissolved in pH 8.8 phosphate buffer (10 cm^3 0.1 mol dm^{-3}). *N*-Ethyl-4-dihydronicotinamide (50 mg, 3.2 × 10^{-4} mol) or *N*-benzyl-4-dihydronicotinamide (50 mg, 2.3 × 10^{-4} mol) was added and the solution stirred for 1.5 h. The substrate α,α,α-trifluoroacetophenone (100 mg, 5.75 × 10^{-4} mol) was then added and the solution incubated for 47 h **20** or 75.5 h **19**.

The second experiment used was pyridinium papain **20** (20 mg, 8 × 10^{-7} mol) and *N*-ethyl-4-dihydronicotinamide (50 mg, 3.2 × 10^{-4} mol). Following the same procedure completely, a control experiment was also done in the absence of **19** or **20**.

After the reactions, the solutions were extracted with (3 × 10 cm^3). Products were determined by HPLC (Spherisorb 10 ODS2) eluting with acetonitrile–water (1:2). Ethyl pyruvate was reduced under the same conditions.

Acknowledgements

We thank the Carnegie Trust for the Universities of Scotland for a Research Studentship (D. J. A.), the European Community for a Science Stimulation Grant (R. A.) and The Royal Society (S. O. O.).

References

1 G. M. Whitesides and C.-H. Wong, *Angew. Chem. Int. Edn., Engl.*, 1985, **27**, 622; J. B. Jones, *Tetrahedron*, 1986, **42**, 3351; C. J. Suckling in *Enzyme Chemistry, Impact and Applications*, ed. C. J. Suckling, Chapman & Hall, London, 1990, pp. 95–170.

2 D. W. Ribbons, *Science Progress*, 1990, **74**, 205; S. J. C. Taylor, D. W. Ribbons, A. M. Z. Slawin, D. A. Widdowson and D. J. Williams, *Tetrahedron Lett.*, 1987, **28**, 6391; S. C. Taylor in *Opportunities in Biotransformations*, eds. L. G. Copping, R. E. Martin, J. A. Pickett, C. Bucke and A. W. Brench, Elsevier Applied Science, Barking, 1990, pp. 170–176.
3 For example, Y. Gao, R. M. Hanson, J. M. Kluender, S. Y. Ko, H. Masumune and K. B. Sharpless, *J. Am. Chem. Soc.*, 1987, **109**, 5765; E. J. Corey, N. Imai and H. Y. Zhang, *J. Am. Chem. Soc.*, 1991, **113**, 728.
4 R. A. Lerner, S. J. Benkovic and P. G. Schultz, *Science*, 1991, **252**, 659; C. J. Suckling, M. C. Tedford, L. H. Bence, J. J. Irvine and W. H. Stimson, *Bioorg. Med. Chem. Lett.*, 1992, **2**, 4952; A. I. Khalaf, G. R. Proctor, C. J. Suckling, L. H. Bence, J. J. Irvine and W. H. Stimson, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1475.
5 E. T. Kaiser, *Angew. Chem., Int. Edn. Engl.*, 1988, **27**, 913.
6 T. Kokubo, S. Sassa and E. T. Kaiser, *J. Am. Chem. Soc.*, 1987, **109**, 606.
7 B. L. Iverson and R. A. Lerner, *Science*, 1989, **243**, 1184.
8 A. W. Schwabacher, M. I. Weinhouse, M. T.-M. Auditor and R. A. Lerner, *J. Am. Chem. Soc.*, 1989, **111**, 2344; A. G. Cochran and P. G. Schultz, *Science*, 1990, **249**, 781; A. G. Cochran and P. G. Schultz, *J. Am. Chem. Soc.*, 1990, **112**, 9414.
9 A. G. Cochran, T. Phar, R. Sugasawara and P. G. Schultz, *J. Am. Chem. Soc.*, 1991, **113**, 6670.
10 N. T. Nashed and E. T. Kaiser, *J. Am. Chem. Soc.*, 1986, **108**, 2710.
11 K. Yamamura and E. T. Kaiser, *J. Chem. Soc., Chem. Commun.*, 1976, 830.
12 I. MacInnes, D. C. Nonhebel, S. T. Orszulik, C. J. Suckling and R. Wrigglesworth, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2771.
13 R. Favilla, A. Mazzini, A. Fava and P. Cavatorta, *Eur. J. Biochem.*, 1980, **104**, 217; R. Favilla, P. Cavatorta, A. Mazzini and A. Fava, *Eur. J. Biochem.*, 1980, **104**, 229.
14 J. B. Givert, V. E. Price and J. P. Greenstein, *J. Biol. Chem.*, 1949, **180**, 473; K.-H. van Pée, O. Salcher and F. Lingens, *Annalen.*, 1981, 223; B. F. Crowe and F. F. Nord, *J. Org. Chem.*, 1950, **15**, 688; W. S. Fones, *J. Biol. Chem.*, 1953, **204**, 323.
15 J. B. Jones in *Asymmetric Synthesis*, vol. 5, ed. J. D. Morrison, Academic Press, New York, 1985, pp. 309–344.
16 B. L. Vallee, J. A. Dupley, T. L. Coombs and H. Neurath, *J. Biol. Chem.*, 1960, **235**, 64; J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, 1960, **235**, 390; J. E. Coleman and B. L. Vallee, *J. Biol. Chem.*, 1961, **236**, 2244; A. Schaffer and D. S. Auld, *Biochemistry*, 1986, **25**, 2476; R. J. DeKoch, D. J. West, J. C. Cannon and N. D. Chasteen, *Biochemistry*, 1974, **13**, 4347.
17 H. B. Gillespie and H. R. Snyder, *Org. Syn.*, Coll. Vol. 2, 1943, 489.
18 K. Kumar, D. J. Phels and P. R. Carey, *Can. J. Chem.*, 1978, **56**, 232.
19 B. D. Vineyard, W. S. Knowles, M. J. Sabacky, G. L. Bachman and D. J. Weinkauff, *J. Am. Chem. Soc.*, 1977, **99**, 5946.
20 S. A. Latt and B. L. Vallee, *Biochemistry*, 1971, **10**, 4263.
21 R. C. Rosenberg, C. A. Root and H. B. Gray, *J. Am. Chem. Soc.*, 1975, **97**, 21.
22 F. A. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry*, 4th edn., Wiley, New York, 1980.
23 D. C. Rees, M. Lewis and W. N. Lipscomb, *J. Mol. Biol.*, 1983, **168**, 367.
24 W. C. Wolsey, C. A. Reynolds and J. Kleinberg, *Inorg. Chem.*, 1963, **2**, 463.
25 J. A. Hinson and R. A. Neal, *J. Biol. Chem.*, 1972, **247**, 7106; K. Dalziel and F. M. Dickinson, *Nature*, 1965, **206**, 255; C. S. Tsai and D. S. Sher, *Arch. Biochem. Biophys.*, 1980, **199**, 626.
26 M. F. Dunn and S. A. Bernhard, *Biochemistry*, 1971, **10**, 4569; M. Kuwada, S. Howie and Y. Ogura, *J. Biochem.*, 1980, **88**, 859.
27 C. S. Tsai, *Biochem. Biophys. Res. Commun.*, 1979, **86**, 808; *Arch. Biochem. Biophys.*, 1982, **213**, 636.
28 E. Vis and H. G. Fletcher, Jr., *J. Am. Chem. Soc.*, 1957, **79**, 1182.
29 S. Hanessian and J. Baraub, *Carbohydr. Res.*, 1977, **59**, 261.
30 B. A. Arbuzov and E. N. Ukhvatova, *J. Gen. Chem. USSR.*, 1959, **29**, 501.
31 G. Wulff and G. Röhle, *Angew. Chem., Int. Edn. Engl.*, 1974, **13**, 157.
32 J. Smrt, *Tetrahedron Lett.*, 1973, 4727; W. S. Zielinski and J. Smrt, *Collect. Czech. Chem. Commun.*, 1974, **39**, 2483; S. N. Michailov and J. Smrt, *Collect. Czech. Chem. Commun.*, 1975, **40**, 2353.
33 D. J. Aitken, L. Rees, C. J. Suckling and H. C. S. Wood, *Tetrahedron Lett.*, 1986, **27**, 3417.
34 B. Capon, *Quart. Rev.*, 1964, **18**, 65.
35 S. Sicsic, P. Durand, S. Langrené and F. LeGoffic, *Eur. J. Biochem.*, 1986, **155**, 403.
36 J. T. Slama, C. Radziejewski, S. R. Oruganti and E. J. Kaiser, *J. Am. Chem. Soc.*, 1984, **106**, 6778.
37 K. M. Shokat, C. J. Leumann, R. Sugasawara and P. G. Schultz, *Angew. Chem., Int. Edn. Eng.*, 1988, **27**, 1172.
38 R. Feeney, A. R. Clarke and J. J. Holbrook, *Biochem. Biophys. Res. Commun.*, 1990, **166**, 667; C. H. Vonderosten, A. J. Sinksey, C. F. Barbas, R. L. Pedersen, Y. F. Wang and C.-H. Wong, *J. Am. Chem. Soc.*, 1989, **111**, 3924; C.-H. Wong, S. T. Chen, W. J. Hennen, J. A. Bibs, Y. F. Wang, J. L.-C. Liu, M. W. Pantoliano, M. Whitlow and P. N. Bryan, *J. Am. Chem. Soc.*, 1990, **112**, 945.
39 R. K. Ness, H. W. Diehl and H. G. Fletcher, Jr., *J. Am. Chem. Soc.*, 1954, **76**, 763.
40 R. A. Earl and L. B. Townsend in *Nucleic Acid Chemistry*, part 1, eds. L. B. Townsend and R. S. Tipson, Wiley, New York, 1978, pp. 203–205.
41 E. Rosenburg and S. Zanenhof, *J. Biol. Chem.*, 1962, **237**, 1040.
42 R. E. Thiers, *Methods Anal. Biochem.*, 1957, **5**, 273.
43 A. Darbe in *Practical Protein Chemistry*, ed. A. Darbe, Wiley-Interscience, Chichester, 1986, pp. 294–295.
44 K. B. Brocklehurst, J. Carlsson, M. P. Kieistan and E. M. Crook, *Biochem. J.*, 1973, **133**, 573.
45 J. W. Bridges and R. T. Williams, *Nature*, 1962, **196**, 59.
46 K. B. Sharpless and J. R. Verhoeven, *Aldrichimica Acta*, 1979, **12**, 63.

Paper 2/05015C
Received 18*th September* 1992
Accepted 19*th October* 1992