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Occurrence of an allosteric transition in the modification of papain with L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]-indole-2-carboxamide

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ABSTRACT

When papain was reacted with L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide at pH 8.0, inactivation occurred accompanied by modification of Cys-25 in the active site. Plots of pseudo first-order rate constants against the reagent concentrations yielded an anomalous sigmoidal curve, suggesting that papain responded to this reagent in an allosteric manner. This is supported by the fact that the presence of a moderate concentration (a twenty-fold molar excess) of N^{α}-acetyl-L-tryptophanamide over papain accelerated the inactivation.

INTRODUCTION

Oxidation of N^{α}-acetyl-L-tryptophanamide or N^{α}-acetyl-L-tryptophan methyl ester with N-bromosuccinimide in phosphate buffer at about pH 8.0–9.0, or with *tert.*-butyl hypochlorite in triethylamine-buffered methylene chloride–dimethylformamide (1:1) gives tricyclic dihydropyrrolo[2,3-*b*]indoles characterized by an absorption maximum at 308 nm (ε = 12 200 mol^{-1} l cm^{-1}) [1,2]. These dihydropyrrolo[2,3-*b*]indoles are stable above pH 7.0, but below this pH they are rapidly converted to oxindole derivatives with an absorption maximum at 250 nm [3]. Recently, 2,3-dihydropyrrolo[2,3-*b*]indoles were found to be efficiently converted to 2-(alkylthio)-L-tryptophan derivatives (ε = 11 000 mol^{-1} l cm^{-1} at 290 nm) when reacted with thiols such as 2-mercaptoethanol, L-cysteine and glutathione in ammonium hydrogencarbonate buffered aqueous dioxane or dimetylformamide at 55°C (Fig. 1) [4]. No amino acids other than cysteine reacted with 2,3-dihydropyrrolo[2,3-*b*]indoles at about pH 8.0–9.0. These findings suggest that 2,3-dihydropyrrolo[2,3-*b*]indoles might be useful for the chemical modification of thiol groups of proteins. In the work reported here, an attempt was made to modify the active site cysteine residue of papain with L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide. Inactiviation occurred in an allosteric manner, suggesting that papain, a monomeric enzyme, behaves allosterically against compounds with a hydrophobic nature.

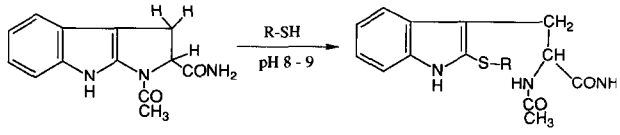

Fig. 1. Modification of a cysteine residue with L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide.

EXPERIMENTAL

Materials

L-1-Acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide was synthesized by the oxidation of N^{α}-acetyl-L-tryptophanamide with *tert.*-butyl hypochlorite according to the method described by

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Ohno *et al.* [2]. Benzoyl-L-arginine ethyl ester (BAEE) was purchased from the Peptide Institute, Osaka, Japan. Other reagents used were of the best grade available.

Papain

Papain (twice crystallized), obtained from Sigma (St. Louis, MO, USA), was further purified by affinity chromatography on a mercurial Sepharose column [5]. Active papain was prepared by the activation of mercuri-papain with 2-mercaptoethanol or L-cysteine at pH 8.0 (0.1 *M* phosphate buffer) followed by separation of the protein from the reducing agent on a Sephadex G-25 column. The preparation had a thiol content of 1.04 mol/mol protein when determined with 5,5′-dithio*bis*(2-nitrobenzoic acid) [6]. The papain concentration was determined with $E^{1\%}_{1cm} = 25.0$ at 280 nm.

ACTIVITY ASSAY

The activity of papain was assayed with 10 m*M* BAEE as substrate. The liberated acids were titrated on a Radiometer (Copenhagen, Denmark) RTS-5-titration assembly at pH 8.0 and 37°C.

Amino acid analysis

This was conducted on a Hitachi (Osaka, Japan) 835 amino acid analyser.

Reaction of amino acid mixture with L-1-acetyl-2,3-dihydropyrrolo[2,3-b]indole-2-carboxamide

An equimolar mixture of nineteen amino acids other than L-cysteine was reacted with a twenty-fold molar excess (per each amino acid) of L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide at pH 8.0 (0.1 *M* phosphate buffer) for 12 h and analysed on an amino acid analyser.

Reaction of papain with L-1-acetyl-2,3-dihydropyrrolo[2,3-b]indole-2-carboxamide

To papain solution ($1.8 \cdot 10^{-5}$–$2.3 \cdot 10^{-5}$ *M*) in 0.1 *M* phosphate buffer (pH 8.0) containing 1 m*M* EDTA was added a solution of L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide in dimethyl sulphoxide to give 50-, 100-, 133-, 150 and 200-fold molar excesses. The reactions were carried out at 37°C and at intervals aliquots were withdrawn and assayed for activity. Pseudo first-order rate constants were obtained from the slopes of semi-logarithmic plots of relative activities *versus* time. The reactions were also carried out in the presence of various concentrations of N^{α}-acetyl-L-tryptophanamide.

RESULTS AND DISCUSSION

It was established that the reaction of L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide and L-cysteine produces N^{α}-acetyl-L-2-(S-cysteinyl) tryptophanamide [4]. When a mixture of nineteen amino acids other than L-cysteine was incubated with a twenty-fold molar excess (per each amino acid) of L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide at pH 8.0 for 12 h and analysed on an amino acid analyser, all amino acids were quantitatively recovered. The fact that 2,3-dihydropyrrolo[2,3-*b*]indole can specifically react only with L-cysteine provides a basis to apply this reagent for the chemical modification of thiol group(s) of proteins. Papain, a popular thiol protease, was selected for modification with 2,3-dihydropyrrolo[2,3-*b*]indole.

When papain was exposed to more than 100-fold molar excesses of L-1-acetyl-2,3-dihydropyrrolo-[2,3-*b*]indole-2-carboxamide at pH 8.0 and 37°C, it was rapidly inactivated. No reaction was observed when the inactivated papain was mixed with 5,5′-

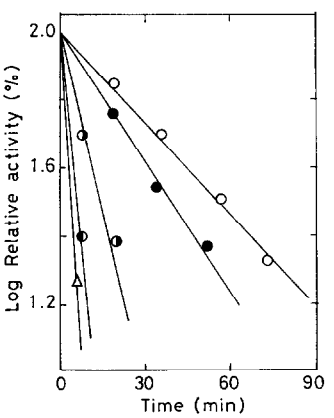

Fig. 2. First-order plot for inactivation of papain with various concentrations of L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide at pH 8.0 (0.1 *M* phosphate buffer containing 1 m*M* EDTA) and 37°C. Papain 2.0×10^{-5} *M*. Reagent concentrations: 1.0 m*M* (○), 2.0 m*M* (●), 2.7 m*M* (◑), 3.0 m*M* (◐) and 4.0 m*M* (△).

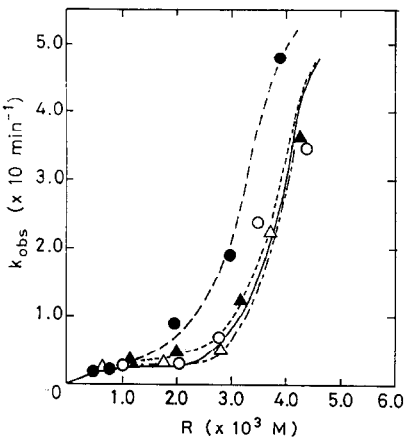

Fig. 3. Plots of pseudo first-order rate constants (k_{obs}) for inactivation of papain ($1.8 \cdot 10^{-5}$ *M*) *versus* L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide concentrations in the absence (○) and presence of 20- (●), 70- (△) and 100-fold molar excesses (▲) of N^{α}-acetyl-L-tryptophanamide in terms of the papain concentration at pH 8.0 and 37°C.

dithio*bis*(2-nitrobenzoic acid) at pH 8.0, indicating that the reagent reacted with Cys-25 in the active site of papain.

Fig. 2 shows first-order plots for the inactivation of papain when exposed to 50-, 100-, 133-, 150- and 200-fold molar excesses of L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-caboxamide at pH 8.0 and 37°C. From the inclination of these straight lines, pseudo first-order rate constants (k_{obs}) were determined. The relationship between k_{obs} values and the reagent concentrations is shown in Fig. 3. An unusual sigmoidal curve was obtained (indicated by the open circles). When the reagent concentration was less than a 100-fold molar excess over papain, inactivation proceeded at a slow rate. However, when the reagent concentration was increased above a 150-fold molar excess, the inactivation rate was greatly enhanced. Such a kinetic behaviour of papain towards L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide could be ascribed to the allosteric nature of papain. This appears to be induced by the hydrophobic nature of the reagent.

In an attempt to convert this sigmoidal curve to less sigmoidal or to a saturation curve, N^{α}-acetyl-L-tryptophanamide, which is hydrophobic in nature, was introduced into the reaction mixture. When a twenty-fold molar excess of N^{α}-acetyl-L-tryptophanamide over papain was added, the curve shifted to the left, as shown in Fig. 3 (indicated by the closed circles). This means that N^{α}-acetyl-L-tryptophanamide induces a conformational change in papain through hydrophobic interactions to enhance the reactivity of Cys-25 towards L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide. However, in the presence of 70- or 100-fold molar excess of N^{α}-acetyl-L-tryptophanamide (indicated by open and closed triangles, respectively), the curves obtained were sigmoidal, similar to that obtained in the absence of the additive. It is likely that N^{α}-acetyl-L-tryptophanamide at such high concentrations inhibits papain by orienting itself to the active site and thus preventing the access of Cys-25 to the reagent.

This work shows that papain behaves like oligomeric allosteric proteins against L-1-acetyl-2,3-dihydropyrrolo[2,3-*b*]indole-2-carboxamide. Papain appears to respond allosterically to hydrophobic compound(s), although it exists in a monomeric form.

REFERENCES

1 M. Ohno, T. F. Spande and B. Witkop, *J. Am. Chem. Soc.*, 90 (1968) 6521.
2 M. Ohno, T. F. Spande and B. Witkop, *J. Am. Chem. Soc.*, 92 (1970) 343.
3 M. Ohno, T. F. Spande and B. Witkop, *J. Org. Chem.*, 39 (1974) 2635.
4 M. Ohno, S. Tanaka, T.-C. Shieh and T. F. Spande, *J. Org. Chem.*, 49 (1984) 5069.
5 L. A. E. Sluyterman and J. Wijdenes, *Biochim. Biophys. Acta*, 200 (1970) 593.
6 G. L. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.