AN ENZYMATIC APPROACH TO THE LABELING OF TRYPTOPHAN RESIDUES IN PEPTIDES AND PROTEINS

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1. Introduction

One of the main obstacles to the analysis of peptide action at the cellular level is the design of radioactive labeling procedures which do not significantly alter the biological activity of the labeled peptide. Of the two isotopes commonly used to obtain compounds of high specific radioactivity (^{3}H, ^{125}I), only tritium labeling produces true tracers without modification of physical or chemical properties. However, since suitable unsaturated or halogenated precursors for catalytic reduction with tritium gas can only be obtained in a few exceptional cases, iodination is the method commonly employed. The resulting difficulties associated with the production of an analog have been well documented for insulin and glucagon [1–5], and even more clearly for angiotensin and posterior pituitary hormones [6–9].

The recent discovery and isolation of a novel bacterial hemoprotein enzyme, named indolyl-3-alkane α-hydroxylase [10], suggested an alternative and partially enzymatic approach to labeling of proteins and peptides. The enzyme catalyzes the formation of an unsaturated intermediate (3-alkylidene indoline) which can be isolated if both the carboxy and α-amino group of tryptophan are protected by substitution [11].

In this paper we present a summary of new observations that bear on the reaction mechanism of the enzyme and describe its application to the formation of an aliphatic double bond specifically in tryptophan residues of peptides and proteins. This double bond reacts readily with ICl and iodine and it can also be reduced catalytically with hydrogen and hydrogen isotopes.

2. Materials

The chemicals used in this work were obtained from the following sources: all indole derivatives, gastrin related peptide, adrenocorticotrophic hormone (ACTH), egg white lysozyme and myoglobin from Sigma; Sephadex gels from Pharmacia; urea and dithiothreitol from Biorad; hydrogenation catalysts and all buffer chemicals from Merck (Darmstadt). The enzyme was isolated from *Pseudomonas* and purified to homogeneity as in [10]. The α-neurotoxin from *Naja naja siamensis* was purified as in [12]. Plasminogen activator was a gift of Drs E. Reich and H.-D. Schleuning, The Rockefeller University, New York, Synacthen (Tetracosactid hexaacetate) which structurally is identical with ACTH 1-24 was a gift of Ciba-Geigy, Switzerland. ACTH (from Sigma) yielded several bands by polyacrylamide gel electrophoresis; the chemical purity was approx. 70–80%.

3. Results and discussion

3.1. N-*Acetyl-L-tryptophanamide substrate*

To define optimal experimental conditions for modifying peptides, we first investigated the enzyme's action on model compounds, in particular *N*-acetyl-L-tryptophanamide. The results, which will be published in detail elsewhere, extend the data [10,11,13, 14] but focus mainly on the reactivity of the reaction products rather than on the mechanism of the enzymatic reaction.

The enzymatic reaction is accompanied by changes in ultraviolet absorption [11,13] and fluorescence [15] of the indole substrates. With *N*-acetyl-L-tryptophanamide as substrate a new absorption band with maximum at 333 nm was formed (fig.1). No isosbestic points were detected [11,13] even when the enzyme was removed from the reaction mixture by gel permeation chromatography. The ultraviolet spectrum of the product(s) depended upon the pH at which the reaction occurred and upon the buffer composition and concentration in a rather complex manner [15,16]. For example, the $A_{333\ nm/276\ nm}$ varied from about 0.5 for reaction in 0.05 M sodium acetate buffer, pH 3.5, to a maximum of about 2.2 M for 0.05 M potassium phosphate buffer, pH 7.0. When incubated with the enzyme, the typical indole fluorescence emission band with maximum (uncorrected) at 352 nm progressively diminished and was replaced by a weak new emission band with maximum at 396 nm (for excitation at 333 nm) [15]. Throughout the reaction, the enzyme's intrinsic fluorescence spectrum with maximum at 323 nm was not changed indicating that its tryptophan residues in their rather hydrophobic environment were not also substrates of the enzyme [15].

Using ultraviolet absorption and fluorescence as monitoring parameters we have analyzed the solvent conditions of the enzymatic reaction and have studied iodination and catalytic hydrogenation of the result-

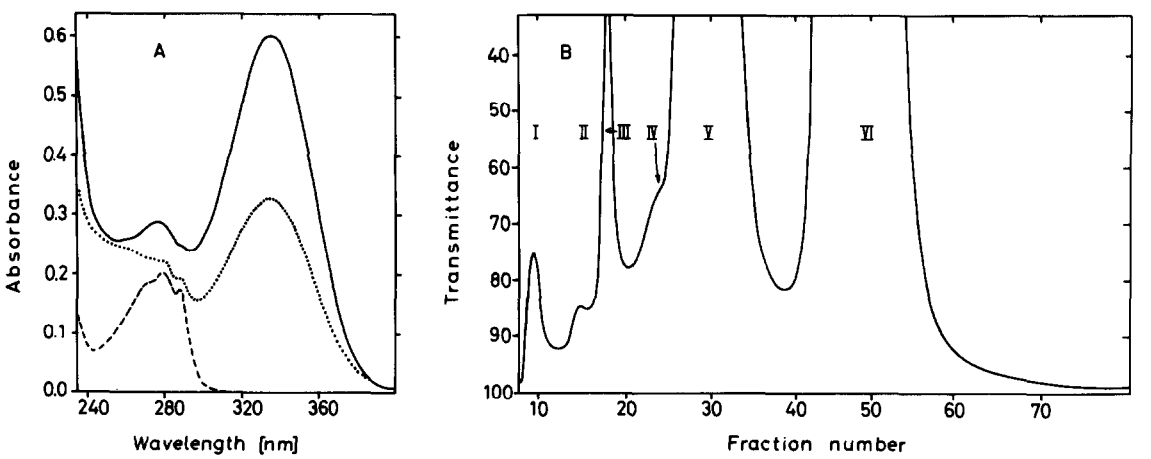

Fig.1A. Ultraviolet absorption spectra of *N*-acetyl-L-tryptophanamide, its enzymatic reaction product and reaction of the latter with ICl. *N*-Acetyl-tryptophanamide (3.6×10^{-5} M) was reacted at 25°C with 3 μg of purified enzyme in 1 ml 0.05 M potassium phosphate buffer, pH 7.0. Spectra were recorded before addition of enzyme (– – –) and after completion of the reaction (——). The spectral ratio $A_{333/276}$ remained constant even after removal of the enzyme by gel permeation chromatography using Sephadex G-25. Reaction with ICl: Before addition of ICl to an aliquot of peak VI of the chromatographic separation of fig.1B, the reaction mixture had the same A_{333} as (——). ICl, 2×10^{-8} mol, were added to the didehydrotryptophan derivative in 1 ml of 0.05 M potassium phosphate buffer, pH 7.0 (. . .). Sample and reference cuvette were not matched in this experiment. Fig.1B. Gel permeation chromatography on Sephadex G-25 of the reaction mixture. *N*-acetyltryptophanamide, 12 mg, were reacted at 25°C with 25 μg of purified enzyme in total vol. 4.05 ml 0.025 M Tris–HCl buffer, pH 7.2. After 30 h the reaction mixture was applied to a column (1.5 × 30 cm) of Sephadex G-25 equilibrated with H_2O. Fractions of 50 drops were taken. Peak I (void vol.) contained the enzyme while all other products were of low molecular weights. Peaks III and IV showed ultraviolet A_{295} max, peak V contained unreacted *N*-acetyltryptophanamide and peak VI, the major reaction product, was identified as 3-alkylidene-indoline derivative by its spectral properties and reactivity to ICl.

ing products. For the latter studies we have separated the enzyme from the products by Sephadex G-10 or G-25 chromatography (fig.1); this procedure led to the resolution of several minor products both from unreacted *N*-acetyl-L-tryptophanamide and from the main reaction product, the latter being retarded significantly more than the unchanged substrate. These were our findings:

(i) The enzyme was active over a wide range of pH values, in 8 M urea and in 0.25–1.0% sodium dodecylsulfate solution at pH 7.

(ii) The main reaction product did not react with sodium borohydride [13,15] but did react with both ICl and I_2 [15]. Reaction with ICl resulted in an almost complete disappearance of the long wavelength ultraviolet absorption band without reappearance of the original spectrum of *N*-acetyl-L-tryptophananamide (fig.1). Titration of excess ICl with sodium dithionite showed that there existed approx. 0.8 mol olefinic double bond/mol reaction product [15].

(iii) The reaction product was susceptible to catalytic hydrogenation [13]. Reduction of the double bond occurred readily with PdO as catalyst but only much more slowly with $PtO_2 . xH_2O$ or Pd-charcoal [15]. Hydrogenation was accompanied by the disappearance of the long-wavelength absorption band and simultaneous reappearance of the spectrum of the initial substrate *N*-acetyl-L-tryptophanamide (fig.1). However, we never succeeded in completely converting the enzyme's reaction products into the initial substrate, probably because of the occurrence of side reactions (fig.1).

The enzyme acted by the same mechanism on a variety of indole-3-alkanes including 3-indole propionic acid, 3-indoleacetic acid [15] and skatole [11]; this was also so for a large number of tryptophan containing di- and tri-peptides [13,15]. While we were able to establish the formation of an aliphatic double bond in all of these by reaction with ICl and/or catalytic hydrogenation, the reaction usually progressed further as indicated by consecutive spectral shifts. Stable didehydrotryptophan derivatives were only obtained if both the carboxyl and α-amino groups were substituted [15].

The above data confirm the formation of an olefinic double bond during the enzymatic reaction [11,13]

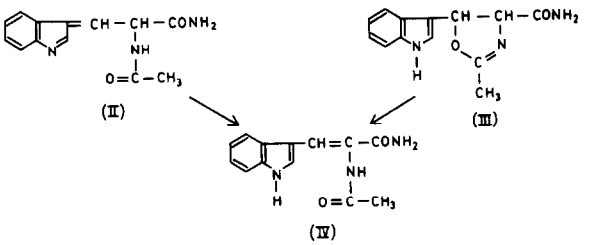

Fig.2. Chemical structure of products identified after reaction of indolyl-3-alkane α-hydroxylase with *N*-acetyltryptophanamide. The 3-alkylidene indoline (II) has been shown by mass spectrometry and NMR to be the initial product of the enzymatic reaction at pH 5.5 [11]. In addition several other products were identified in the reaction mixture, another major one being the oxazoline derivative III [11]. Upon addition of acid to the reaction mixture one major product, the α–β-didehydro derivative IV, was formed [11] which is structurally identical with the unsaturated product isolated [13].

and lend support to the proposal [11] that compound II (fig.2) but not IV [13] is the initial intermediate of the enzymatic reaction.

3.2. *Peptide substrates*

Once the action of indolyl-3-alkane hydroxylase on *N*-acetyl-L-tryptophanamide had been analyzed, its application for modifying peptides and proteins became feasible. These studies showed that peptides are generally much less efficient substrates than simpler indole derivatives and, in some cases, did not react at all. A prominent example of the latter case was cobra α-neurotoxin [12] which neither reacted in buffer at pH 7 nor pH 5, nor in 8 M urea or 1% SDS. However, after reduction of some of its disulfide bonds by mercaptoethanol, the single tryptophan residue became susceptible to enzymatic attack in 8 M urea and yielded a reaction product which, after reoxidation of the cysteines, readily reacted with ICl.

In contrast to the apparently rather rigidly structured α-neurotoxin (5 S–S bridges, 72 amino acids), adrenocorticotrophic hormone (ACTH, 39 amino acids, no S–S bridges) was a good substrate of the enzyme. To achieve a reaction rate with ACTH similar to that with *N*-acetyl-L-tryptophanamide required approx. 20-times more enzyme (substrate concentration in both cases 5×10^{-5} M, phosphate buffer, pH 7.0). As with *N*-acetyl-tryptophanamide, a new

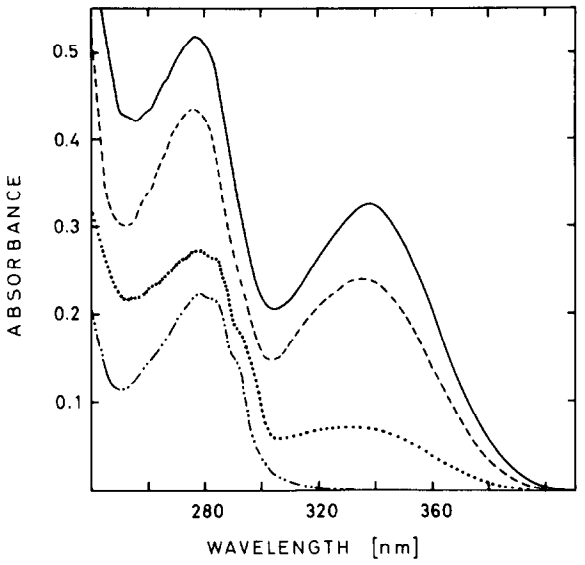

Fig.3. Ultraviolet absorption spectra of ACTH, its enzymatic reaction product and the product after partial hydrogenation. ACTH (5.5×10^{-5} M) was reacted at 25°C with 50 μg purified enzyme in 1.1 ml 0.05 phosphate buffer, pH 7.0 until completion (3 h). Spectra were recorded before addition of enzyme (–· ·–) and at the end of the reaction (——). Catalytic hydrogenation was carried out in the cuvette with 1 mg PdO as catalyst. (– – –) Spectrum after 20 min reduction, (. . . .) spectrum after 90 min reduction. Spectra were measured in presence of enzyme and are not corrected for the absorbance of the enzyme.

absorption band with maximum at 336 nm developed, $A_{336/275}$ was, however, much smaller than for the former product. The intrinsic indole fluorescence of ACTH was also abolished. As described for the didehydrotryptophan derivatives, when the unsaturated product was reacted with ICl and I_2, at pH 7, there were large changes in the indole absorption spectrum, but since ACTH contains a histidine and two tyrosine residues besides tryptophan, these are likely also to have been altered, and we have in fact established that extensive product heterogeneity is obtained on iodination [15]. In contrast, as seen in fig.3, a partial catalytic reduction of the enzymatic reaction product using PdO as catalyst gave minor heterogeneity with over 80% reduced product consisting of a single species inseparable by column chromatography from authentic ACTH. Other effective substrates found so far include: poly(D,L-tryptophan), tryptophylalanine, tryptophyltyrosine, and L-tryptophyl-methionyl-aspartyl-phenylalanineamide. The gastrin related peptide *tert*-amyloxycarbonyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalanine amide was a good substrate for the enzyme and the reaction product was easily hydrogenated to regenerated starting material. Chicken egg-white lysozyme reacted only partially (less than 20%) under non-reducing conditions. The largest polypeptide tested to date was myoglobin, which reacted best in 8 M urea.

4. Conclusion

Indolyl-3-alkane α-hydroxylase is a novel catalyst suitable for the preparation of didehydrotryptophan residues in peptides and proteins. The resulting double bond reacts readily with ICl or I_2; it is also susceptible to catalytic hydrogenation and deuteration and, by inference, tritiation. Since tritium is obtainable in carrier-free form, specific radioactivities of 56 Ci/mmol reacted tryptophan residue should be obtainable with little difficulty [17] and at low cost.

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