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Short Communications

Action of the Bacterial Proteinase, Subtilisin B, on "Native" Hemoglobin A

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The commonly held opinion that "native" or "undenatured" proteins are not digested by enzymes is difficult to document by investigations that have used sensitive modern methods. Indeed, many proteins require such complex and protracted methods of isolation that the identity of the molecule with its native state may truly be questioned. The present investigation was designed to study the effect of enzymes on "native" or "undenatured" hemoglobin A through the medium, first, of the pH-stat.[1] Hemoglobin may be isolated under milder conditions than most proteins (conditions, at least, which do not alter its most vital property of reversible dissociation with oxygen) and, hence, presumably it is in a state near to that of the native or undenatured protein. The most significant results were obtained with subtilisin A [2] and its near relative, subtilisin B,[3,4] (previously designated as Novo bacterial proteinase) from a related strain of *Bacillus subtilis*.

Whole oxyhemoglobin A was prepared by the method [5] except that the preparation was dialyzed against distilled water. Subtilisin B (Lot Ch31C) was a preparation from the Novo Pharmaceutical Company, Copenhagen. Subtilisin A was taken from a preparation that had been made by methods previously described [2]. Conditions of the reaction were: pH 8.00, temperature 31.5°C, a specific gaseous atmosphere in the reaction vessel, 118 mg of hemoglobin in 5 ml of water, and 1 mg of subtilisin B unless subtilisin A is specified. The specific gaseous atmosphere was achieved by passing a gas over the reaction mixture at a rate of 10 ml per min under one of three conditions: (1) nitrogen for 2 h at pH 8.00 prior to the addition of enzyme and throughout the reaction period, (2) nitrogen, but the pH was brought to 8.00 immediately and enzyme was added, and (3) a mixture of oxygen and nitrogen in the proportions in air to exclude carbon dioxide. A Radiometer pH-stat with a Dich recorder [1] was used to record the uptake of base as a function of time after the addition of enzyme. After the reaction had reached the desired state of completion, any remaining protein was precipitated by the addition of an equal volume of 20 % trichloroacetic acid. The precipitate was filtered off and selected samples were dinitrophenylated. The non-protein material

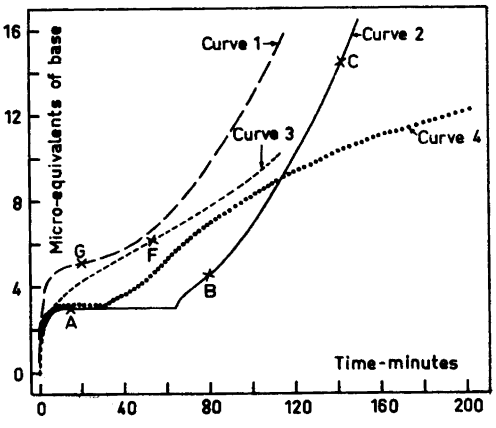

Fig. 1. Action of subtilisin B on hemoglobin A. The figure reproduces the uptake of base as a function of time as recorded by the pH-stat. In some experiments, the action of the enzyme was continued for longer periods: thus, point D on Curve 2,26.6 μequiv. at 165 min; point E on Curve 2, 145 μequiv. at 665 min; point H on Curve 1, 21.0 μequiv. at 140 min. For further details, see text.

in the filtrate was investigated by Kjeldahl analysis in order to determine the quantity, by unidimensional paper electrophoresis, and by "fingerprinting"[6].

Fig. 1 depicts typical pH-stat tracings, which have been superimposed at the point of addition of enzyme. The uptake of base described by Curve 1 resulted under condition (1) above, in which the enzyme is acting on deoxygenated hemoglobin.[7] Curve 2 shows the uptake under condition (2); oxyhemoglobin is first present but deoxygenates in the course of the experiment. Under condition (3), which produces Curve 3, oxyhemoglobin is maintained in this form during the experiment.

In the initial stages the curves follow rather different courses, but later all show an increasing rate. The enzyme under conditions (1) and (3) is acting on a definite derivative of hemoglobin, but under condition (2) the form of the derivative changes in the course of the reaction. The cessation of uptake in Curve 2 between 10 and 65 min results because the deoxygenating oxyhemoglobin is acting as its own pH-stat: because reduced hemoglobin is a weaker acid than oxyhemoglobin [7], the molecule itself combines with the hydrogen ions that normally would be titrated. The differences in Curves 1 and 3 presumably reflect a difference in the attack of the enzyme on the two derivatives. The results shown in Fig. 1 may be obtained reproducibly with samples of oxyhemoglobin that have been stored at 5° C for periods of one to two weeks. Older samples may show more bizarre effects. Presumably, older solutions would have had greater opportunity to denature, and hence the action of the enzyme should be more rapid. The reverse is the case. For example, Curve 4 resulted to a greater or lesser degree under essentially condition (2) when old samples were studied: the rate of reaction actually fell off in the later stages. The explanation of this type of behavior is not obvious.

In order to gain an insight into the nature of the reaction, the material not precipitated by trichloroacetic acid was further studied. The reaction was stopped at various points that are indicated by the point on the curve and the letter adjacent in Fig. 1. The non-precipitable nitrogen is essentially a linear function of the uptake of base; at point C, about 10 % was non-precipitable. A sample corresponding to point B, as expected from the above explanation of this curve, contains more nitrogen in solution than would be expected from the uptake of base. Portions which contained equivalent quantities of non-precipitable nitrogen were taken from available samples and submitted to paper electrophoresis at pH 3.5 in pyridine-acetic acid buffer. The results from all samples noted in Fig. 1 (except E, which was not then available) were identical both in movement of the spots and in the relative amounts of the various spots; indeed, the same results were obtained with a sample from the action of subtilisin A. Samples from points B, D, E were selected for more extensive investigation by »fingerprinting»[6]. The fingerprints of samples D and E were essentially indistinguishable and that of sample B differed only in small details. Fig. 2a is representative of the results. In a further comparison, a sample of heat-denatured [6] hemoglobin was treated with enzyme until the uptake of base approximated that of sample D; the time required to achieve the same uptake of base was 4 min with the heat-denatured sample as compared to 165 min with sample D. A comparison of the fingerprints of sample D and of heat-denatured hemoglobin is presented in Fig. 2. Appreciable differences are apparent.

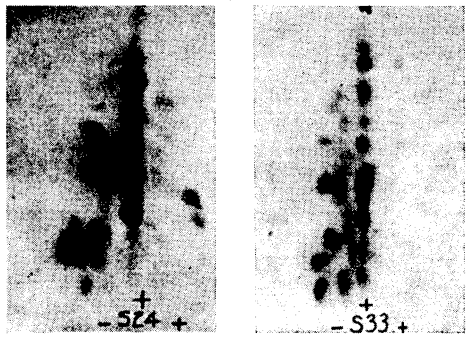

Fig. 2. "Fingerprints" of non-precipitable material from the action of subtilisin B on (a) undenatured and (b) heat-denatured hemoglobin A.

The trichloroacetic acid precipitate from sample D was dinitrophenylated. The DNP-protein was hydrolyzed for 15 min in refluxing 6 N hydrochloric acid as previously described[8]. The N-terminal DNP-peptides were extracted, identified, and quantitatively estimated. The DNP-pep-

tides agreed both in kind and quantity with those [8,9] that would be obtained from hemoglobin that had not been treated with enzyme.

The results of the present investigation bear upon several as yet unanswered questions about the action of proteolytic enzymes on proteins. (1) They show that certain enzymes act vigorously on hemoglobin, a protein which is probably as "native" or "undenatured" as any. (2) They indicate that the type of reaction is the so-called "one-by-one" type [10]; that is, at any time after the action of the enzyme has begun, a molecule has either reacted to the fullest possible extent or it has not reacted at all. The identity of the non-precipitable products at various stages of reaction and the normal nature of the N-terminal DNP-peptides of the trichloroacetic acid precipitate are strong evidence for this conclusion. (3) They show that, if an enzyme must denature a protein prior to cleaving it or if it reacts only with the denatured protein of a reversible equilibrium between denatured and undenatured protein [11], then, the denaturation is of a different type than that produced by heat because the products from "undenatured" and heat-denatured hemoglobin differ.

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Kinetics of the Reaction between β-Sulfinylpropionic Acids and Iodide Ion in Acid Solution

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The rate of the reaction: $RSOR' + 2\,H^+ + 2\,I^- \rightarrow RSR' + I_2 + H_2O$ where $R = HOCOCH_2CH_2$-, $R' = HOCOCH_2CH_2$-, and $CH_3CH_2CH_2$-, has been studied at 25°C in solutions of perchloric acid and perchloric acid-sodium perchlorate. With hydrogen- and iodide ions in excess the reaction is kinetically of the first order, *i.e.*, one obtains the rate expression:

$$k = \frac{1}{t} \ln \frac{a}{a-x}$$

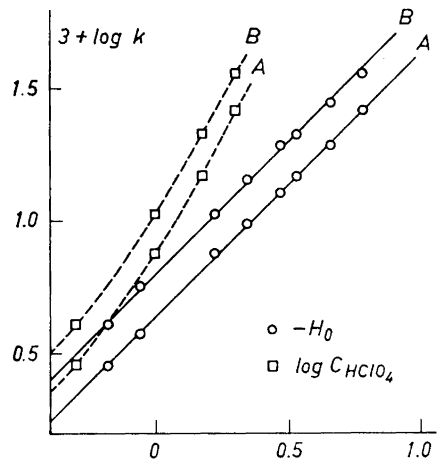

Fig. 1. Log k as a function of $-H_0$ (unbroken lines) and of log C_{HClO_4} (dashed lines) for the reaction for A and B, respectively, with iodide ion in acid solution at 25°C. (The dashed curves refer to perchloric acid solutions without any added sodium perchlorate.)