Esterase Activity of Streptolysin-O

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SUMMARY

Purified streptolysin-O, from group-C streptococci, H46A, whose identity with NAD-glycohydrolase (EC. 3.2.2.5) was reported earlier, has been shown to hydrolyse 4-nitrophenyl-esters. With 4-nitrophenylacetate as substrate, K_m was 6.6×10^{-5} M; turnover number was 10000 mol substrate/mol enzyme/min and optimum pH was 7.8 to 8.5. Esterase activity was activated by SH-compounds and inhibited by diisopropylphosphorfluoridate, mercuric salts and oxidizing agents. The effects of these agents on esterase activity were similar to their effects on the haemolytic activity of streptolysin-O protein so that both catalytic processes may be accomplished by the same active site.

INTRODUCTION

Numerous micro-organisms produce cytolytically active substances which cause lysis of mammalian cells in various tissues. Some are also haemolysins, which liberate haemoglobin from red blood cells. There are two different kinds of haemolysins (Todd, 1938). One group is reversibly inactivated by oxidation, the other is not affected by oxygen or sulphhydryl compounds. Among the oxygen-labile haemolysins the following are best known: pneumolysin, tetanolysin, cereolysin and streptolysin-O. These lysins seem to resemble each other very closely but their biochemical nature and the mechanism of haemolysis is not clear, partly because their instability has complicated isolation and purification. However, appreciable progress has been made in the purification of streptolysin-O (Halbert & Auerbach, 1961; Alouf & Raynaud, 1968). Following its isolation in a homogeneous form (Fehrenbach, 1971*a*) the activities of streptolysin-O and NAD-glycohydrolase (EC. 3.2.2.5) were reported to be catalytic functions of the one protein molecule (Fehrenbach, 1971*a, b*). However, the idea that NAD-glycohydrolase might be responsible for the lysis of red cells could not easily be reconciled with the present view of the membrane structure of erythrocytes. Thus, a correlation between glycohydrolase and haemolytic activity could be made only if the enzyme possessed a broader specificity.

It was therefore important to investigate whether streptolysin-O from *Streptococcus* C H46A possessed hitherto undetected hydrolytic activity, which could explain the mechanism of enzymatic haemolysis.

METHODS

Enzyme. Streptolysin-O, (NAD-glycohydrolase; EC. 3.2.2.5) from group-C streptococci, Laboratory strain H46A, was kindly supplied from Behringwerke Marburg/Lahn, G.F.R., and purified according to the method reported earlier (Fehrenbach, 1971*a*). Purified enzyme preparations of sp. act. 7.4×10^2 units of esterase activity of streptolysin-O (SLO-esterase)/mg protein, were stored at -20°C . Immediately before use, the enzyme was thawed and activated for 30 min at 30°C with 0.6 mM-2-mercaptoethanol.

Reagents. Unless otherwise stated, analytical grade reagents were used. 2-Mercaptoethanol, Puriss., was bought from Fluka AG, Buchs SG, Switzerland. Diisopropylphosphorfluoridate and 4-(chloromercuri)-benzoic acid, sodium salt, were obtained from Schuchardt, München, G.F.R. Acetic acid 4-nitrophenylester (for biochemical use) L(+)-cysteine, and acetonitrile were purchased from Merck AG, Darmstadt, G.F.R.

Substrate solution. Prior to use, 4-nitrophenylacetate was recrystallized twice from dried ether. The crystals were dried at 4°C over P_2O_5 under reduced pressure in the dark, until the solvent was completely removed. 4-Nitrophenylacetate was dissolved in acetonitrile to 3 mM and this substrate solution, which is stable at 4°C for several hours without appreciable hydrolysis, was used to determine the activity of SLO-esterase. The final concentration of acetonitrile in the reaction mixture did not exceed 3%, v/v.

Kinetic measurements. Photometric measurements were performed in a M4Q II Zeiss spectrophotometer (Carl Zeiss, Oberkochen, G.F.R.) connected with a Siemens Polycomp recorder, Type K 105/027. Unless otherwise stated, reactions were started by the addition of substrate. The reaction mixture was placed in quartz cuvettes, 1 cm light path, and maintained at 30°C. The kinetic data were derived from the zero-order part of the photometric trace during the initial reaction period.

Enzyme assay. A 2.8 ml sample of 0.1 M-tris HCl buffer, pH 8.0, containing 0.6 mM-mercaptoethanol, 0.1 ml enzyme solution (approximately 10^{-8} to 10^{-9} M) and 0.1 ml of 0.11 mM-4-nitrophenylacetate were placed in a 1 cm light path cuvette. Readings were also taken of a control from which enzyme was omitted to correct for non-enzymatic hydrolysis of 4-nitrophenylacetate. In cases where enzyme or substrate concentrations varied from the above, exact data are reported in the legends of the corresponding figures. One enzyme unit (1 U) of SLO-esterase is defined as that catalytic activity which causes an extinction difference at 400 nm of 0.001/min, at 30°C, in a 1 cm light path cuvette.

Protein estimation. Protein was measured either by the method of Folin & Ciocalteu (1927), or by the photometric method outlined by Warburg & Christian (1942), in the latter case being calculated from the following equation, $E_{280} \times 0.91 = \text{mg protein/ml, 1 cm light path}$.

Assay of haemolytic activity. Sheep erythrocytes were washed 3 times with 1 mM-sodium phosphate-buffered isotonic NaCl solution, pH 7.5. A precalculated volume of the washed erythrocyte suspension was diluted in the cuvette with isotonic NaCl solution to give an extinction of 0.100 at 546 nm after complete haemolysis in 3.0 ml 0.1% (w/v) Na_2CO_3 solution.

The reaction mixture of total volume 3.0 ml, in 1 cm light path cuvettes, contained 0.1 ml of SLO-esterase, 0.2 ml of the adjusted erythrocyte suspension and 2.7 ml isotonic buffer. When inhibition of the rate of haemolysis by diisopropylphosphorfluoridate was investigated the inhibitor was dissolved in the isotonic buffer. The mixture was then incubated with the enzyme for 20 min at 30°C.

Haemolysis was initiated by the addition of the pre-incubated erythrocyte suspension and the rate of haemolysis followed continuously. The change in extinction at 546 nm was followed using a Vitatron photometer coupled to a chart recorder (Vitatron, Dieren, Holland). Inhibition values were obtained by comparison with a control which contained the enzyme and the erythrocytes (as 'substrate') but no inhibitor. All reactions were followed to completion but inhibition values were calculated from the linear portion of the curve describing the rate of haemolysis.

Fig. 1. Dependence of the rate of hydrolysis of 4-nitrophenylacetate on enzyme concentration. The reaction was carried out in 0.1 M-tris HCl-buffer, pH 8.00, containing 0.6 mM-2-mercaptoethanol, and 0.11 mM-4-nitrophenylacetate.

RESULTS

Hydrolysis of 4-nitrophenylesters

Streptolysin-O catalysed the hydrolysis of 4-nitrophenylacetate. The homologous esters of propionic and butyric acids were also hydrolysed, but at much slower rates. Under given conditions the rate of substrate cleavage was a linear function of the enzyme concentration (Fig. 1). Values plotted on the ordinate in Fig. 1 were determined from the zero-order part of the spectrophotometric trace where $S \gg K_m$.

When the reaction rate was plotted as a function of the substrate concentration at constant enzyme concentration (6 nM), a typical hyperbolic curve was obtained (Fig. 2). The curve resembles that of human serum esterase which however has a greater K_m value with 4-nitrophenylacetate as substrate (Huggins & Lapides, 1947). The K_m for this substrate was 6.6×10^{-5} M as shown in Fig. 2; this value was also confirmed by a Lineweaver/Burk plot. With the given maximal velocity (see $v/2$, Fig. 2) and with the mol. wt of streptolysin-O taken as 60000 (Fehrenbach, 1969), the turnover number was approximately 10000 mol substrate/mol enzyme/min. Certain kinetic 'constants' such as K_m values, turnover number and optimum pH may vary considerably from substrate to substrate (Delory & King 1943; Schonheyder & Volqvartz, 1945). However, the determination of these constants for SLO-esterase seemed to be essential for the development of a method for the biochemical estimation of SLO-esterase activity.

pH optimum

With other conditions maintained constant, the rate of hydrolysis of 4-nitrophenylacetate ($\mu\text{mol/min}$) was measured at pH values from 6.0 to 9.5, the concentration of 4-nitrophenol produced in the assay medium being calculated from the extinction at 400 nm using a molar extinction coefficient $E_{400 \text{ nm}}^{200}$ of $9.89 \text{ cm}^2 \mu\text{M}^{-1}$.

Fig. 3 shows that the maximum rate of hydrolysis was obtained between pH 7.8 and 8.5; with pH values greater than 9.0 or less than 6.5 the rate of hydrolysis was considerably

Fig. 2. Effect of substrate concentration on the rate of enzymatic hydrolysis of 4-nitrophenylacetate. The reaction system described in Fig. 1 was employed with approx. 6 mM enzyme.

Fig. 3. Effect of pH on the rate of enzymatic hydrolysis of 4-nitrophenylacetate.

Fig. 4. The effect of 2-mercaptoethanol, 4-chloromercuribenzoate and hydrogen peroxide on the rate of enzymatic hydrolysis of 4-nitrophenylacetate. Untreated enzyme ●—●; enzyme treated with 0.6 mM-2-mercaptoethanol, ■—■; enzyme treated with 0.1 mM-4-chloromercuribenzoate, ○—○; enzyme treated with 0.1 mM-H₂O₂, ×—×, for 30 min at 30°C in 0.1 M-tris HCl buffer, pH 8.00; 4-chloromercuribenzoate treated enzyme after 6 h dialysis against 10 mM-L-cysteine, ▲—▲.

lowered, though the enzyme was stable over the range pH 4.0 to 9.5. In this respect streptolysin-O differs from streptococcal proteinase, which cleaves phenyl and 4-nitrophenyl esters at comparable rates above pH 5.0 (Teh-Yung Liu, Nomura, Jonsson & Wallace, 1969).

Activation of SLO-esterase by SH-compounds

Since streptolysin-O belongs to the group of SH-dependent haemolysins the behaviour of SLO-esterase in the presence of SH-compounds was investigated. Fig. 4 shows the effect on SLO-esterase activity of 2-mercaptoethanol, H₂O₂ and 4-chloromercuribenzoate, activity being assayed as the rate of change in extinction at 400 nm due to the release of 4-nitrophenol. The reaction velocity increased threefold after treatment with 2-mercaptoethanol. In contrast, either 4-chloromercuribenzoate or H₂O₂ completely destroyed esterase activity.

Following 4-chloromercuribenzoate treatment, dialysis for 6 h against 10 mM-cysteine did not fully restore activity.

Maximum activation of SLO-esterase was achieved with 0.6 mM-2-mercaptoethanol after 30 min at 30°C. This concentration of 2-mercaptoethanol did not promote non-enzymatic autolysis of 4-nitrophenylacetate. Therefore, as with haemolysis of red blood cells (Stock & Uriel, 1961; Todd, 1938), the cleavage of nitrophenylesters was activated by thiol compounds and inactivated by oxidizing reagents.

Sensitivity of SLO-esterase to organophosphorous compounds

Organophosphorous compounds can be useful reagents with which to characterize esterases (Aldridge 1954). The effect of diisopropylphosphofluoridate (DFP) on the activity of SLO-esterase is shown. In Fig. 5 DFP (10⁻⁶ M) caused 50% inhibition. Since the mol. wt of SLO-esterase is 60000, the molar ratio of enzyme/inhibitor for 10⁻⁸ M-DFP was approximately 1, with 10⁻⁸ M-DFP the rate of hydrolysis was only decreased by 5%; with 10⁻⁷ M-DFP more than 70% of the original activity was retained. Thus, the stoichiometry of the

Fig. 5. Inhibition of hydrolysis of 4-nitrophenylacetate (●—●) and of haemolysis of red blood cells (▲—▲) by various concentrations of diisopropylphosphofluoridate. Initial (i.e. 100 %) activity of SLO-esterase = 182 U/ml.

reaction indicates that phosphorylation of the active site of the enzyme may not occur. In this case inhibition may be reversible, and indeed the inhibition of SLO-esterase by DFP could be overcome by short-term dialysis. This raises the question whether DFP functions as a true substrate or as a competitive inhibitor when competing with 4-nitrophenylacetate for the active site of the enzyme.

Fig. 5 also shows that haemolysis is influenced by DFP, since above a certain concentration further increase in DFP concentration was associated with a decrease in the rate of cell lysis. Inhibition of haemolysis was calculated from the slope of the linear part of the appropriate lysis curves, though it is widely accepted (Ikezawa, 1963) that haemolysis exhibits more complex kinetics and has been shown to be at least a biphasic process. Thus, both the haemolysis of red blood cells as well as the SLO-esterase activity were inhibited by DFP.

DISCUSSION

Because of the lack of an absolute criterion for the homogeneity of polypeptides, the question arises whether the breakdown of different chemical bonds by an enzyme is a function of one or more enzyme species. Therefore, the different catalytic functions of an enzyme have sometimes been interpreted as the action of distinct molecular species, (Carlson, Kellner, Bernheimer & Freeman, 1957; Halbert, 1958; Petersen, Kröger & Rothauwe, 1961). Although the multiple specificities of streptolysin-O are functions of the one macromolecular species (Fehrenbach, 1971*a, b*), in general the possibility exists that enzymes may be characterized *in vitro* by catalytic properties which are irrelevant to their true function *in vivo*. Streptolysin-O may be an example, although it is not generally accepted that cytolysis is catalysed by an enzyme (Bernheimer, 1970). But, together with my earlier findings, the present investigation provides evidence for a correlation between the different catalytic functions of streptolysin-O and its cytolytic activity. This is indicated by the molecular homogeneity of streptolysin-O (Fehrenbach, 1971*a, b*) and by the fact that the haemolytic and esterase activities responded similarly to 2-mercaptoethanol (activation), 4-chloromercuribenzoate, H_2O_2 and DFP (reversible inhibition). The inhibition of enzymatic activity by DFP may resemble the inhibition of streptolysin-O induced haemolysis

that is caused by some phospholipids of human sera (Petersen, Nowak, Thiele & Urbaschek, 1966). This leads to speculation concerning the substrate role of phospholipids. However, on the basis of present knowledge, streptolysin-O may be characterized as a 'hydrolase' which catalyses the breakdown of C-O and C-N⁺ ≤ bonds.

The findings regarding the inhibition and activation of esterase, glycohydrolase and haemolytic activities suggest that these multiple specificities may be associated with one protein molecule and possibly one active site. Though the true substrate of streptolysin-O is not yet known, it is conceivable that the cleavage of C—O and/or C—N⁺ ≤ bonds plays an essential role in streptolysin-O catalysed haemolysis.

This concept of an enzymatic cytolysis immediately makes a number of related problems more amenable to investigation; these include the kinetics of haemolysis, inhibition of red blood cell lysis by several organic compounds, disagreement of values of the enzyme-anti-enzyme reaction (streptolysin-O-anti-streptolysin-O reaction) and denaturation of the enzyme protein. However, the streptolysins from other sources may differ in their behaviour from those produced by the H46A strain investigated here.

The rapid assay for streptolysin-O-esterase activity in purified preparations developed here is clearly defined, convenient to use and more specific than the traditional lysis test.

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