Identity of Streptolysin-O and NAD-Glycohydrolase (EC 3.2.2.5)

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The homogeneity of purified NAD-glycohydrolase (EC 3.2.2.5) from Group C streptococci was demonstrated by analytical ultracentrifugation, disc electrophoresis and immunochecmical analysis. Together with earlier characterization procedures the data show that the enzyme is homogenous in molecular seize, molecular form and electrophoretic charge. In addition, the enzyme has been shown to possess strong hemolytic activity which could be activated by thiol compounds. It was concluded from the analytical data that NAD-glycohydrolase- and Streptolysin-O-activities are catalytic functions of one protein molecule. Therefore the lysis of red blood cells by Streptolysin-O may be regarded as an enzymatic process catalyzed by an NAD-glycohydrolase.

ROBERT KOCH first reported (1884) that vibrio bacteria could lyse red blood cells. Soon after the introduction of the blood agar technique ¹ this phenomenon was found to be common to many microorganisms. Within this group, bacterial hemolysins from streptococci were most intensively studied because of their significance in human streptococal diseases ².

Neill and Mallory ³ and Herbert and Todd ⁴-⁶ demonstrated the sensitivity to oxygen of one of the extracellular hemolysins associated with streptococci from Group A, C and G-strains, known as "Streptolysin-O", SLO (O = oxygen). Because of its considerable instability, Streptolysin-O has never been obtained in pure state until now, in spite of many attempts ⁷-¹².

Similar instability was also detected in NAD-glycohydrolase, NADase, (EC 3.2.2.5), a streptococcal exoenzyme, described earlier ¹³-¹⁵ and isolated in 1970 ¹⁶. Apart from this reported instability, it was shown that purified NADase possessed hemolytic activity which could be activated by sulfhydryl compounds. Thus a molecular correlation between NAD-glycohydrolase and Streptolysin-O was expected ¹⁶.

Material and Methods

The protein concentration, the NAD-glycohydrolase- and Streptolysin-O-activities were measured according to the methods described earlier ¹⁶.

Analytical ultracentrifugation

The sedimentation velocity pattern of purified NAD-glycohydrolase from Group C streptococci, H 46 A, were studied in 0.1 M Tris/HCl buffer, pH: 8.0, at 20 °C in a Beckman Model E analytical ultracentrifuge (rotor An-D) 59 780 rpm. The protein concentration was 11.4 mg/ml, the specific activity: \( SA = 1.1 \times 10^8 \) units NADase/mg prot. The Schlieren photographs taken at 8 min intervals, reproduced in the upper row of Fig. 1, were taken after the centrifuge had reached maximum speed (\( \leq 60° \)). As shown in the lower row of Fig. 1, photographs were taken after 95 min at 16 min intervals, Schlieren angel \( \approx 70° \).

Disc electrophoresis

The disc electrophoresis of purified NAD-glycohydrolase was carried out in 6% polyacrylamid gel, in 0.1 M Tris/HCl buffer, pH: 8.0, 4 °C, 5 mA/tube, 1.5 h. The specific activity of the enzyme was: \( SA = 2.7 \times 10^2 \) units NADase/mg prot; 100 \( \gamma \) protein/tube.

Immunodiffusion

Immunodiffusion of purified NAD-glycohydrolase was carried out in 0.8% Agarose in 0.1 M Tris/HCl buffer,

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pH: 7.8. NAD-glycohydrolase was detected with pooled human γ-globulin, 16%, code Nr. 3324, Behringwerke Marburg, Lahn, Germany. In the range of equivalence 70 γ of the enzyme protein were applied to the whole (Fig. 3 a).

A modified immunodiffusion technique on a blood agar slide was used to demonstrate the hemolytic activity of the purified enzyme as well as to demonstrate the inhibition of the hemolytic activity of NADase by different immune sera (Fig. 3 b). To the central slit of the blood agar slide 65 μl of NADase (5 mg prot/ml, specific activity: $\text{SA} = 1.5 \times 10^8$ units NADase/ml $\pm 1.3 \times 10^8$ hemolytic units/ml) previously dialysed in isotonic NaCl solution containing 10 mM cysteine, were applied. The inhibition of hemolysis was tested with three different immune sera: Anti-NADase, 4200 anti-units; anti-Streptolysin-O, 3486 anti-units/ml; pooled human γ-globulin, 16%, code Nr. 3324.

Results

NADase has been isolated from culture supernatant of β-hemolytic streptococci, Group CH46 A*, as reported earlier 16. During the purification procedure the quotient, $Q$, of the specific activities (in terms of $\text{mg prot.} / \text{SA NADase}$)

\[
\text{SA NADase} = \frac{\text{unit NADase}}{\text{mg prot.}} \quad \text{or} \quad \text{SA SLO} = \frac{\text{unit SLO}}{\text{mg prot.}} \quad Q = \frac{\text{SA SLO}}{\text{SA NADase}},
\]

indicating the efficiency of a separation step, was followed. The constancy of the $Q$-values (see Table) suggests that an efficient separation of both SLO- and NADase-activities did not occur. It seemed therefore essential to investigate the pure state of NADase. However, because of the lack of an absolute criterion of homogeneity, a variety of characterization procedures were employed. These methods usually characterise macromolecules on the basis of various physicochemical properties i.e. molecular form, size or electrophoretic charge. A convenient method for the study of molecular state of NADase is the determination of its sedimentation properties in the analytical ultracentrifuge. It can be seen from Fig. 1 that the sedimentation profile of NADase shows a monodisperse distribution of the component within the time of run. Ignoring a possible concentration dependence of the sedimentation constant, the calculated value of $S$ was: 3.42, which is in good agreement with earlier observations 17.

The electrophoretic properties of native NADase were examined by disc electrophoresis in 6% polyacrylamid gel. Under the given conditions, the enzyme migrated as a single band towards the anode (Fig. 2). This result was also obtained when the electrophoresis was carried out at different pH values (6.7 – 8.7) thus confirming the charge homogeneity of the native enzyme.

* The crude, lyophilized material was supplied from Behringwerke, Marburg/Lahn, Germany.

\begin{tabular}{|c|c|c|c|}
\hline
 & crude material & AS-pre-precipitation & chromatography on G-100 & chromatography \\
\hline
\text{SA NADase} & $5.3 \times 10^2$ & $5.5 \times 10^3$ & $1.2 \times 10^3$ & $1.3 \times 10^3$ \\
\text{SA SLO} & $5.3 \times 10^2$ & $7.5 \times 10^3$ & $1.9 \times 10^4$ & $1.7 \times 10^4$ \\
\text{mg prot./ml} & 44.9 & 43.6 & 4.2 & 2.4 \\
\text{purification} & 0 & 1.1 & 2.3 & 2.5 \\
\text{SA SLO} & 13.8 & 13.6 & 15.6 & 13.1 \\
\text{SA NADase} & & & & \\
\hline
\end{tabular}

Tab. I. Specific activities of NADase and SLO and constancy of specificities during purification.
Furthermore, it is of particular interest that immunological techniques can be employed for the demonstration of the pure state of NAD-glycohydrolase. As a protein antigen, NADase and SLO are known to induce the synthesis of specific antibodies in mammals during streptococcal infections. These antibodies were shown to react with NADase and SLO in an antigen-antibody reaction (enzyme-antienzyme reaction) thus inhibiting the catalytic function of the antigen.

As demonstrated in Fig. 3a with the outherlony technique, NADase (X) shows one precipitation line only when tested against human γ-globulin (central well). At the same time the hemolytic activity of purified NADase was demonstrated using a modified blood agar technique. As can be seen from Fig. 3b, a clear hemolytic zone emerges from the central slit where the enzyme has been applied. However, the hemolytic activity is inhibited at the positions 1, 2, 3 but not at 4 which contained isotonic buffer only. This inhibition of hemolysis is due to the inactivation of NADase by specific antibodies derived from different immune sera including human-γ-globulin 3, anti-NADase 1, and anti-SLO sera 2.

Although different immune sera containing different antibody concentrations and species such as anti-Streptolysin-O or anti-NAD-glycohydrolyase antibodies were used, the same degree of inhibition of hemolysis was demonstrated.

**Discussion**

The purification and crystallization of NAD-glycohydrolase has provided first evidence for a chemical correlation between NAD-glycohydrolase from Group C streptococci and the well known hemolytic toxin Streptolysin-O. This finding was also in agreement with earlier observations. However, to confirm the association of NAD-glycohydrolyase and Streptolysin-O-activity with one molecular species it was essential to investigate the pure state of NADase by a variety of characterization procedures. Thus, because of the lack of an absolute criterion of homogeneity for polypeptides the pure state of NADase was investigated by analytical gel-permeation chromatography, analytical hydroxylapatite chromatography, density gradient centrifugation as well as by analytical ultracentrifugation, disc electrophoresis and immunochemical analysis. The data show the homogeneity of the purified enzyme in molecular size, molecular form and electrophoretic charge. The enzyme is a potent hemolytic agent and the hemolytic activity is activated by thiol compounds. In addition, the hemolytic activity of NADase is completely inhibited by anti-Streptolysin-O as well as by anti-NAD-glycohydrolyase immune sera, though different antibody concentrations and species should be expected. Thus it was concluded from the data that both NAD-glycohydrolyase and Streptolysin-O activities are catalytic functions of one protein molecule. Therefore, the hemolysis of red blood cells by Streptolysin-O, from Group C streptococci, H 46 A, may be regarded as an enzymatic process catalyzed by an NAD-glycohydrolyase of broad substrate specificity.

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