Reactions of Essential Sulfhydryl Residues of Dehydrogenases with Nicotinamide-(S-Methylmercury-Thioinosine) Dinucleotide

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Sulfhydryl Groups, Methylmercury Containing Inactivator, Coenzyme Analogue

Nicotinamide-(S-methylmercury-thioinosine) dinucleotide was formed by reaction of nicotinamide-(6-thiopurine) dinucleotide with methylmercury chloride. The compound exhibits coenzyme properties in the test with LDH ($K_m = 1.5 \times 10^{-4}$ M, $V_{max} = 12500$) and LADH ($K_m = 1.7 \times 10^{-4}$ M, $V_{max} = 27$) and inactivates YADH and GAPDH. From incubations with LDH and LADH the mercury containing coenzyme could be regained by column chromatography. The compound seems to be qualified for the X-ray structure analysis of the coenzyme-enzyme complex for some dehyrogenases based on the proportion of the heavy metal.

Introduction

Cysteine residues frequently take part in enzymatic catalysis. These residues easily react with mercury compounds, causing total loss of the catalytic properties of the enzyme [1].

The participation of SH-groups at the formation of the active center of $NAD^{\oplus}$-dependent dehydrogenases was found. The natural coenzyme $NAD^{\oplus}$ is replaceable by the analogue nicotinamide-(6-thiopurine) dinucleotide, without considerable influence in regard to the catalytic properties of the enzyme [2].

Materials and Methods

Enzymes and coenzyme: $NAD^{\oplus}$, LADH, YADH, LDH and GAPDH were obtained from Boehringer & Soehne, Mannheim.

For the synthesis of nicotinamide-(S-methylmercury-thioinosine) dinucleotide 10 mg nicotinamide-(6-thiopurine) dinucleotide [2] were dissolved in 5 ml 0.2 M potassium phosphate buffer pH 6.5 and 5 mg methylmercury chloride, in 2 ml 90 per cent ethanol, were added. After 10 min the reaction mixture was washed twice with 10 ml ether. The aqueous phase was concentrated to 1 ml at 30 °C in vacuo, applied to a Sephadex G 10 column (130 × 15 cm), and eluted with water. The compound appeared after 140 ml of effluent in a volume of 70 ml, surplus methylmercury chloride appeared after 290 ml.

The solvent was evaporated by lyophilisation. The yield was 12 mg.

$\lambda_{max} = 297$ nm; $\varepsilon = 19\,700$ (pH 6.5); reduced form:
$\lambda_{max} = 297$ nm; $\varepsilon = 19\,400$ (pH 9.5);
$\lambda_{max} = 340$ nm; $\varepsilon = 5\,900$ (pH 9.5).

The phosphate [3] to the ribose [4] ratio was found to be 2 : 1.8.

The *enzymatic reaction* was investigated in 3 ml 0.2 M glycine/NaOH buffer pH 9.5 containing 0.15 mM lithium lactate at 25 °C. The coenzyme concentration was varied between 0.05 mM and 1.5 mM. The reaction was started with 5 µg LDH. In the test with YADH and LADH 1 mM ethanol instead of lithium lactate was added.

The reaction was started by addition of 10 µg LADH or 3 µg YADH. As substrate for GAPDH 0.2 mM glyceraldehyde-3-phosphate in 0.05 M triethanolamine phosphate buffer [5] were used. The reaction was started by addition of 5 µg GAPDH. The rate of the enzymatic reaction was determined as ΔE/min at 366 nm using a photometer recording. In the presence of nicotinamide-(S-methylmercury-thioinosine) dinucleotide the amounts of the enzymes were increased. For the analysis of the *exchange reactions* of the methylmercury residue 40 mg enzyme were dissolved in 4 ml 0.2 M potassium phosphate buffer pH 6.5 containing 0.5 mg nicotinamide-(S-methylmercury-thioinosine) dinucleotide at 25 °C. The enzymatic activity was determined every 15 min. Control incubations con-

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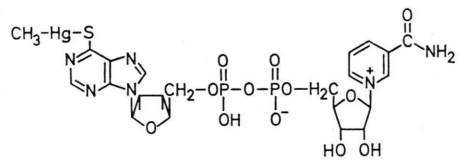
Abbreviations: LDH, lactate dehydrogenase (EC 1.1.1.27); YADH, alcohol dehydrogenase from yeast (EC 1.1.1.1.); LADH, alcohol dehydrogenase of liver (EC 1.1.1.1.); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); $NAD^{\oplus}$, nicotinamide-adenine dinucleotide.

tained NAD® instead of the coenzyme analogue. After 20 hours of incubation the test samples were applied to a Sephadex G 25 fine column (40 × 2 cm) and eluted with 0.2 M potassium phosphate buffer pH 6.5. The coenzyme appeared after 70 ml of effluent in a volume of 20 ml. For the measurement of the *inactivator reaction* 4 mg enzyme were dissolved in 1 ml 0.2 M potassium phosphate buffer pH 6.5. The coenzyme concentration was increased from 1×10^{-4} M to 5×10^{-4} M. The decrease of the enzymatic activity was determined at 37 °C in an interval of 3 min. In case of the inactivation of YADH ethanol until to a concentration of 3 mM was added to the 0.2 M potassium phosphate buffer pH 6.5 to determine the influence of the substrate. *UV-spectra* of the coenzyme fraction were measured with a Cary 14 spectrophotometer. The ratio of nicotinamide-(S-methylmercury-thioinosine) dinucleotide to nicotinamide-(6-thiopurine) dinucleotide was estimated from the extinction at 320 nm and 290 nm at pH 6.5.

Results and Discussion

The thiol group in the coenzyme analogue nicotinamide-(6-thiopurine) dinucleotide reacts with methylmercury chloride to nicotinamide-(S-methylmercury-thioinosine) dinucleotide. Substitution at the position 6 of the purine moiety does not affect the formation of the coenzyme-enzyme complex. The coenzyme analogue should have biological activity. The mercury residue localized in the nonfunctional part of the coenzyme analogue can easily be transferred to other sulfhydryl residues. Specific inactivation could be expected, if an essential sulfhydryl group of the enzyme is in the neighbourhood of the coenzyme binding site. Nicotinamide-(S-methylmercury-thioinosine) dinucleotide shows in electrophoretical analysis one uniform band and appears in a single UV-absorbing fraction after gel chromatography on Sephadex G 10.



The properties of the coenzyme analogue measured in the presence of LDH, YADH, LADH and GAPDH and the corresponding substrates are shown in Table I.

The results are compared with those of nicotinamide-(6-thiopurine) dinucleotide. In the test with LDH there is no difference between the coenzyme function of nicotinamide-(6-thiopurine) dinucleotide and nicotinamide-(S-methylmercury-thioinosine) dinucleotide. In case of LADH the turnover number is reduced by the factor 10. The mercury containing coenzyme shows no activity in the presence of YADH and GAPDH. In all cases the coenzyme analogue is used in excess with regard to the enzyme.

The investigation of the inactivation reaction was carried out by treatment of 1 μmol enzyme subunit with 0.5 μmol of the coenzyme analogue. The incubation is followed by separation and characterization of the coenzyme. The activities of YADH and GAPDH decrease to 50%. In the case of LDH no loss and with LADH 20% loss of catalytic activity takes place. Under the same conditiones LADH and LDH are inactivated by methylmercury chloride without delay. In the presence of various inactivator concentrations a quick inactivation reaction of YADH and GAPDH is observed at first. The amount of this inactivation depends on the concentration of the added inactivator. It is followed by a slow reaction whereby protein precipitates. An exact state-

Table I. Properties of nicotinamide-(S-methylmercury-thioinosine) dinucleotide compared to nicotinamide-(6-thiopurine) dinucleotide.

Enzyme	YADH		LADH		LDH		GAPDH	
	$K_m \times 10^4$ M	V_{max}	$K_m \times 10^4$ M	V_{max}	$K_m \times 10^4$ M	V_{max}	$K_m \times 10^4$ M	V_{max}
Nicotinamide-(S-methylmercury-thioinosine) dinucleotide	inactivated		1.7	27	1.5	12500	inactivated	
Nicotinamide-(6-thiopurine) dinucleotide	5.8	11000	1.7	220	4.5	13000	1.5	12500

V_{max} = μmol substrate/μmol enzyme·x min.

ment concerning an intra- or intermolecular mechanism is not possible. In the presence of the corresponding substrate the rate of inactivation is unchanged.

It is shown that the methylmercury residue of the coenzyme analogue is completely split off in the presence of GAPDH and YADH whereby nicotinamide-(6-thiopurine) dinucleotide originates. The sulfhydryl residues of YADH and GAPDH are easily accessible to the methylmercury group of the coenzyme analogue. After incubation of the mercury containing coenzyme analogue with LDH, 70% of the analogue remains unchanged. Incubation with LADH leads to a mixture containing equal amounts of nicotinamide-(6-thiopurine) dinucleotide and nicotinamide-(S-methylmercury-thioinosine) dinucleotide. The loss of the methylmercury residue in case of the latter enzymes can be explained by a slow reaction between nonessential sulfhydryl groups and the mercury group.

The results of these experiments are in accordance with X-ray analyses of the structure of the enzymes. In case of GAPDH two sulfhydryl residues are localized at the active center [6]. They react easily with SH reagents [7]. In LADH two sulfur atoms of cysteine are covalently connected with a zinc atom in the catalytic center [8]. The essential cysteine residue of LDH is far from the binding site [9]. The structure of YADH is still unknown. By investigations of the primary structure of this enzyme a similarity with the liver enzyme was observed [10]. The essential cysteine residue differs from that of the liver enzyme by its higher ability to react with different SH reagents. The results show that the analogue can be used for the indication of essential sulfhydryl groups near the coenzyme binding site. However, an exact localization of the essential residue with regard to the adenine or nicotinamide binding site is not possible, because the coenzyme can be attached to the active center in a twisted or opposite position [11].

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