A NAD ANALOGUE WHICH CAN BE COVALENTLY BOUND TO DEHYDROGENASES[1]

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Received June 5, 1962

In the course of study of rabbit muscle α-glycerophosphate dehydrogenase an analogue of NAD[3] was prepared in which the nicotinamide moiety was replaced by 4-methyl-5-(β-hydroxyethyl)-thiazole. This analogue, named thiazole-AD, shows the same characteristic behaviour of all quaternary thiazoles in that at moderately alkaline pH values the thiazole ring opens to generate a mercaptan. Since presumptive evidence exists that sulfhydryl groups on the proteins participate in the binding of NAD to a number of dehydrogenases, it was reasoned that this analogue or similar analogues might be useful to label the "active site" of dehydrogenases through the oxidative formation of a disulfide bond.

Thiazole-AD was prepared by the standard exchange method catalyzed by pig brain NAD-ase (Kaplan and Stolzenbach, 1957) in an incubation mixture of the following composition: phosphate buffer, pH 7.5: 3 mmoles; NAD: 200 mgs; 4-methyl-5-(β-hydroxyethyl)-thiazole: 2.8 grams; and pig brain NAD-ase 240 units; in a final volume of 120 ml. Since the analogue is split by pig brain NAD-ase, the reaction was terminated

[1] Supported by an Institutional Grant of the American Cancer Society and National Science Foundation Grant G-14050.

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[3] The abbreviations used are: NAD and NADH, oxidized and reduced nicotinamide adenine dinucleotide (DPN).

when the NAD concentration decreased by 40 per cent (cyanide assay). The residual NAD was destroyed by Neurospora NAD-ase. The analogue is stable to the action of this enzyme. Isolation of the analogue was achieved by chromatography on Amberlite IR-400 in the formate form with a gradient between zero and 1 M formic acid. A product was obtained which had an adenine/ribose/phosphate/thiazole ratio of 1/2/1.8/1. With the use of NAD, labeled in the 8-position of adenine, thiazole-AD-8-(adenine)-C^{14} was prepared in the same manner.

On screening the analogue as a potential inhibitor of a variety of enzymes, it proved to be a reasonably potent competitive inhibitor for a number of enzymes, especially for horse liver alcohol dehydrogenase. This enzyme was selected as a model in view of its ready availability and relatively low molecular weight. Furthermore, a stoichiometry of NAD binding of 2 moles per mole of protein is known (Bonnichsen and Brink, 1955) The analogue was oxidatively linked to this enzyme under the following conditions: sodium carbonate: 30 μmoles; dialyzed enzyme: 20 mgs (approx. 0.3 μmoles); thiazole-AD: 2 μmoles; and $K_3Fe(CN)_6$: 3 μmoles, added in that order. Final volume: 3.0 ml. The reaction was followed spectrophotometrically through the disappearance of ferricyanide at room temperature. The reaction mixture was dialyzed 42 hours against 18 liters dilute acetate buffer pH 6.5 with several changes. The dialyzed solution contained approximately 4.5 moles of phosphate/mole of enzyme. If this solution is then dialyzed against mercaptoethanol, ultraviolet absorbing material is liberated. When enzyme-thiazole-AD-complex was prepared using C^{14}-labeled analogue, a stoichiometry of 1.5-1.9 moles of analogue per mole of enzyme was obtained (Table I). Such protein bound radioactivity was non-dialyzable but became so after treat-

ment of the enzyme with mercaptoethanol (Table II). When labeled enzyme was subjected to a short trypsin digestion with a trypsin/enzyme ratio of 1/7 (weight for weight), the solubilization of the radioactivity was directly proportional to the appearance of trichloroacetic acid-soluble peptides (judged by the Lowry protein method).

Table I

Stoichiometry of binding of thiazole-AD to horse liver alcohol dehydrogenase.

Total counts added	moles analogue added/mole of enzyme	Counts bound to enzyme	moles analogue bound/mole of enzyme
7,000	3.4	1,125	0.52
14,000	6.7	1,800	0.82
28,000	13.3	2,350	1.09
56,000	26.7	3,450	1.60

The oxidation was performed as described in the text except that a varying amount of analogue was used. A molecular weight for horse liver alcohol dehydrogenase of 73,000 was used.

Table II

Linkage of thiazole-AD to alcohol dehydrogenase.

	Activity	
	Inside solution	Outside solution
	cpm/ml	cpm/ml
Control	206	28.5
+ mercaptoethanol	80	80.5

A sample of horse liver alcohol dehydrogenase-thiazole-AD complex was prepared. This was dialyzed in 1 ml against 3.5 ml of 0.1 M phosphate buffer, pH 7.5 in the absence or presence of mercaptoethanol (final concentration of .02 M). After 14 hours dialysis the dialysate and the contents of the dialysis bag were analyzed for radioactivity.

As a further approach to ascertain whether the analogue was bound to the active site, the oxidative coupling of thiazole-AD to alcohol

dehydrogenase was performed in the presence of NAD or NADH. Significant inhibition of binding was observed (Table III).

Table III

Inhibition of binding of thiazole-AD by coenzymes.

Experiment #	Additions	cpm/mg protein	% Inhibition
1	None	18.1	-
	+ NAD	13.7	24.3
2	None	291	-
	+ NAD	217	25.5
	+ NADH	144	50.6

A standard oxidation reaction mixture was used. When added, 4 μmoles of coenzyme were present (either NAD or NADH). In experiment #1 the enzyme analogue complex was isolated by trichloroacetic acid precipitation. The precipitate was washed until no further counts were found in the wash fluid. The residue was then plated and counted. In experiment #2 the reaction mixture was dialyzed for 3 days against several changes of 6 liters each of acetate buffer, pH 6.5. Different analogue preparations were used in the two experiments.

It appears, therefore, that a covalent bond between horse liver alcohol dehydrogenase and thiazole-AD has been achieved through a disulfide linkage. Furthermore, from the stoichiometry data and the inhibition of binding by NAD and NADH, this bond could be at or near the active site. Such an approach to the study of the active site of dehydrogenases with this analogue or similar NAD derivatives could be of general usefulness. Details of the preparation of the analogue will be published in the near future.

REFERENCES

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