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STEREOCHEMISTRY OF THE HYDROGEN TRANSFER TO NAD CATALYZED BY (S)ALANINE DEHYDROGENASE FROM *BACILLUS SUBTILIS*

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Summary

The stereochemistry of the hydrogen transfer to NAD catalyzed by (S)alanine dehydrogenase [(S)alanine : NAD oxidoreductase (EC 1.4.1.1)] from *B. subtilis* was investigated. The label at C-2 of (S) [2,3-^{3}H]alanine was enzymatically transferred to NAD, and the [4-^{3}H]NADH produced isolated and the stereochemistry at C-4 investigated. It was found that the label was exclusively located at the (R) position which indicates that (S)alanine dehydrogenase is an A-type enzyme. This result was confirmed in an alternate way by reducing enzymatically [4-^{3}H]NAD with non labeled (S)alanine and (S)alanine dehydrogenase and investigating the stereochemistry of the [4-^{3}H]NADH produced. As expected, the label was now exclusively located at the (S)position. This proves that (S)alanine dehydrogenase isolated from *B. subtilis* should be classified as an A-enzyme with regard to the stereochemistry of the hydrogen transfer to NAD.

The pioneering work of Vennesland, Westheimer and their coworkers [1,2,3] conclusively demonstrated that NAD and NADP linked dehydrogenases transfer hydrogen stereospecifically to the C-4 position of the nicotinamide ring.

Those dehydrogenases which catalyze transfer to the pro(R) position of the nicotinamide ring were classified as A-enzymes and accordingly these enzymes catalyzing transfer to the pro(S) position were assigned to the B-classification [4].

A relatively large number of dehydrogenases have been investigated regarding the stereochemistry of their hydrogen transfer to the coenzyme and some tentative rules were proposed first by Vennesland [5] and later by Bentley [6] as cited by Popjack [7].

We have recently formulated additional rules which further correlate the

structure of the substrate, the inducible or constitutive nature of the enzyme and its ability to utilize exclusively NAD or NADP, or both coenzymes, with the observed stereochemistry of the hydrogen transfer [16].

One of these proposed rules states that constitutive NAD linked dehydrogenases oxidizing primary or secondary alcohols to aldehydes or ketones, respectively, are always A-type enzymes. In order to extend this rule to NAD-linked dehydrogenases catalyzing the oxidation of primary amines to enamines, we investigated as an example the stereochemistry of the hydrogen transfer to NAD catalyzed by (S)alanine dehydrogenase from *B. subtilis* (L-alanine : NAD oxidoreductase (deaminating) EC 1.4.1.1). This enzyme was shown to be constitutive in nature and is specific for NAD similar to the (S)alanine dehydrogenases isolated from *Bacillus cereus* [9,10] and *B. licheniformis* [10].

Materials and Methods

(S)Alanine dehydrogenase (EC 1.4.1.1) from *B. subtilis*, (S)glutamate dehydrogenase (EC 1.4.1.3) from beef liver, (S)lactate dehydrogenase (EC 1.1.1.27) from rabbit muscle, NAD and (S)alanine were purchased from Boehringer Mannheim Corporation. (S)Lactic acid was obtained from Sigma and (S)glutamic acid from The Matheson Co. The labeled substrates, (S)[2,3-^{3}H]alanine with a specific radioactivity of 42 Ci/mol and [4-^{3}H]NAD with a specific radioactivity of 50 Ci/mol were obtained from New England Nuclear and The Radiochemical Center, respectively.

Enzymatic hydrogen transfer from (S)alanine to [4-^{3}H]NAD

0.75 μmol of [4-^{3}H]NAD with a specific radioactivity of 50 Ci/mol were enzymatically reduced to [4-^{3}H]NADH with 22 μmol of non-labeled (S)alanine and 1.5 units of (S)alanine dehydrogenase in a total volume of 3.4 ml of 0.5 M glycine/hydrazine buffer (pH 9.2). The reaction mixture was incubated at 30 °C and after 60 min when over 95% of the reaction was complete, the NADH produced was isolated and the stereochemistry at C-4 determined as described below. The results are summarized in Table I.

TABLE I

STEREOCHEMISTRY OF THE HYDROGEN TRANSFER FROM (S)ALANINE TO [4-^{3}H]NAD CATALYZED BY (S)ALANINE DEHYDROGENASE

Specific radioactivities (dpm/μmol):			
[4-^{3}H]NAD[1]	[4-^{3}H]NADH[1]	(S)[2-^{3}H]glutamate[2]	NADH[3]
$1.10 \cdot 10^8$	$1.08 \cdot 10^8$	$1.07 \cdot 10^8$	$8.9 \cdot 10^5$

The specific radioactivities of Table I refer to the following steps of the overall reaction scheme:

$$[4\text{-}^3\text{H}]\text{NAD}^1 + \text{(S)alanine} \xrightleftharpoons{\text{(S)AlaDH}} \text{pyruvate} + \text{NH}_3 + [4\text{-}^3\text{H}]\text{NADH}^1$$

$$[4\text{-}^3\text{H}]\text{NADH}^1 + \alpha\text{-ketoglutarate} + \text{NH}_3 \xrightleftharpoons{\text{(S)GDH}} \text{(S)}[2\text{-}^3\text{H}]\text{glutamate}^2 + \text{NAD}^3$$

$$\text{NAD}^3 + \text{(S)lactate} \xrightleftharpoons{\text{(S)LDH}} \text{NADH}^3 + \text{pyruvate}$$

TABLE II

STEREOCHEMISTRY OF THE HYDROGEN TRANSFER FROM (S) [2,3-^{3}H]ALANINE TO NAD CATALYZED BY (S)ALANINE DEHYDROGENASE

Specific radioactivities (dpm/μmol):			
(S) [2,3-^{3}H]alanine	[4-^{3}H]NADH^1	(S)glutamate^2	[4-^{3}H]NADH^3
$2.4 \cdot 10^7$	$1.3 \cdot 10^7$	$1.9 \cdot 10^5$	$1.2 \cdot 10^7$

The specific radioactivities of Table II refer to the following steps of the overall reaction scheme:

$$\text{(S) [2,3-}^3\text{H]alanine} + \text{NAD}^1 \xrightleftharpoons{\text{(S)AlaDH}} [4\text{-}^3\text{H}]\text{NADH}^1 + \text{pyruvate} + \text{NH}_3$$

$$[4\text{-}^3\text{H}]\text{NADH}^1 + \text{NH}_3 + \text{ketoglutarate} \xrightleftharpoons{\text{(S)GDH}} \text{(S)glutamate}^2 + [4\text{-}^3\text{H}]\text{NAD}^3$$

$$[4\text{-}^3\text{H}]\text{NAD}^3 + \text{(S)lactate} \xrightleftharpoons{\text{(S)LDH}} \text{pyruvate} + [4\text{-}^3\text{H}]\text{NADH}^3$$

Enzymatic hydrogen transfer from (S) [2,3-^{3}H]alanine to NAD

$2.3 \cdot 10^{-4}$ μmol (S)[2,3-^{3}H]alanine with a specific radioactivity of 42 Ci/mol was diluted with 0.88 μmol of non-labeled (S)alanine and the hydrogen located at C-2 was transferred enzymatically to NADH with 6 μmol NAD and 1.5 units of (S)alanine dehydrogenase. The reaction was performed in a total volume of 3.1 ml of 0.5 M glycine/hydrazine buffer (pH 9.2) at 30°C. After 60 min of incubation 0.77 μmol NADH were isolated and the stereochemistry at C-4 determined as described below. The results are summarized in Table II. The amount of ^{3}H transferred to NADH indicated that 58% of the original label was present at the C-2 position of the (S)[2,3-^{3}H]alanine used in the experiment.

Isolation of NADH

The NADH produced was isolated by placing the media onto a 1 × 5 cm DEAE-cellulose ion exchange column in the bicarbonate form. Elution with 100 ml of 3.5 mM NH_4HCO_3 displaced (S)alanine and NAD, while the NADH was eluted with 10—15 ml of 0.2 M NH_4CO_3 [12]. This latter eluate was concentrated in vacuo at 40°C, taken up in 2—3 ml water and enzymatically determined with 1.2 μmol of sodium pyruvate and 6 units of (S)lactate dehydrogenase per ml in 1 M NH_4HCO_3 (pH 7 at 25°C) [13].

Analysis of the chirality of the isolated [4-^{3}H]NADH

The ^{3}H content of the (S)-position at C_4 (0.35 to 0.40 μmol [4-^{3}H]NADH) was transferred to (S)glutamate with 1.8 μmol α-ketoglutarate and 3 units (S)glutamate dehydrogenase (a B enzyme) in 1 ml of 1 M NH_4HCO_3 at pH 7 and 25°C. After the reaction had reached equilibrium which took less than 5 min, the enzyme was deactivated by heating for 1 min at 90°C, and the incubation mixture divided into two parts in order to separately determine the specific radioactivities of the concomitantly produced (S)glutamate and NAD. The specific radioactivity of the (S)glutamate produced was determined by diluting 2.8 mmol of non-labeled (S)glutamic acid and, recrystallization from water three times to constant specific radioactivity. In a second experiment, to another aliquot of the original solution containing 0.2 to 0.3 μmol NAD, was added 25 μmol of unlabeled (S)lactate, and the NAD reduced to NADH with 6

units (S)lactate dehydrogenase (an A-enzyme) in 0.5 M glycine/hydrazine buffer of pH 9.5 [14]. The NADH produced was isolated as described above.

Results and Discussion

The results summarized in Tables I and II show that (S)alanine dehydrogenase transfers hydrogen from (S)alanine to the pro (R)-position at C-4 of the nicotinamide ring. It is also shown that labeled [4-^{3}H]NAD when reduced with (S)alanine and the (S)alanine dehydrogenase from *Bacillus subtilis* carries all its label in the (S)-position. These findings support the conclusion that (S)alanine dehydrogenase is an A-enzyme.

The majority of the NAD-linked dehydrogenases working on simple non-phosphorylated substrates and interconverting primary or secondary alcohols and aldehydes or ketones which have been investigated have been found to be of the A-type [2,6,7]. We have advanced the possibility that NAD-linked dehydrogenases working on primary amino groups of non phosphorylated substrates might also be mainly A-type enzymes. The only amino acid dehydrogenase investigated previously is (S)glutamate dehydrogenase, a B-enzyme. This enzyme, however, is not specific for NAD and can work with both coenzymes [15]. Dehydrogenases able to utilize both coenzymes have invariably been found to belong to the B-type [6,7]. As an example of an enzyme specific for NAD and transferring hydrogen from a primary amino group of the substrate we investigated the stereospecificity of the hydrogen transfer from (S)alanine to NAD catalyzed by (S)alanine dehydrogenase from *Bacillus subtilis*. Our expectation that this enzyme would be of the A-type is in agreement with our findings.

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