SPECIFICITY OF RAT LIVER NAD SYNTHETASE

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1. Introduction

In 1958, Preiss and Handler [1, 2] described the biosynthesis of NAD from nicotinic acid in extracts of red blood cells, yeast and rat liver. The final reaction of the biosynthesis was the conversion of deamido-NAD to NAD, which was catalyzed by the enzyme NAD synthetase [deamido-NAD:L-glutamine amido-ligase (AMP) EC 6.3.5.1].

Deamido-NAD + ATP + L-glutamine →
NAD + L-glutamate + AMP + PP_i

In the case of yeast and rat liver enzymes ammonia can substitute for glutamine, but then, at physiological pH values, much higher concentrations of ammonia are required. On the other hand, Imsande [3] and Spencer and Preiss [4] have shown that ammonia rather than glutamine was the nitrogen donor in the reaction catalyzed by *E. coli* NAD-synthetase.

It was found previously that several α-*N*-alkyl derivatives of L-glutamine inhibit the growth of glutamine requiring strains of hemolytic Streptococci [5] and of experimental mouse tumours [6]. It was also found that α-*N*-ethyl and α-*N*-propyl-L-glutamine inhibit ovine brain and rat liver glutamine synthetase *in vitro* [7, 8]. It appeared therefore of interest to study the effect of such derivatives on rat liver NAD synthetase. The effect of carbobenzoxy-L-glutamine and carbobenzoxy derivatives of other amino acids on this enzyme is also investigated.

2. Materials and methods

Deamido-NAD was kindly donated by Dr. N.O.Kaplan, Brandeis University, Waltham, Massachusetts, and also prepared according to Honjo et al. [9].

α-*N*-Alkyl derivatives of L-glutamine were prepared as previously described [5, 7, 10]. They contained less than one percent free glutamine as tested by the colour reaction with aqueous ninhydrin. Carbobenzoxy (Cbz) L-phenylalanine was prepared according to Grassmann and Wünsch [11]. All other Cbz derivatives and L-glutamine were purchased from Fluka A.G., Buchs (Switzerland). Crystalline alcohol dehydrogenase was obtained from Sigma Chemical Company, St. Louis, Mo., nicotinamide and ATP from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Homogenates of rat livers were prepared by the procedure of Melnick and Buchanan [12]. After centrifugation at 70,000 *g* in a Spinco model L centrifuge for one hour, the supernatant fluid was used for the enzyme experiments. Under the conditions of the experiment (pH 7.4) the enzyme preparation were free of NADase activity as tested by the KCN method [13].
Assay procedure: The reaction mixtures contained in 1.5 ml 0.05 M phosphate buffer, pH 7.4 (in μmoles): KCl 56; $MgCl_2$ 5; ATP 4; deamido-NAD 1; nicotinamide 164; L-glutamine, ammonium chloride and Cbz derivatives as indicated in the tables. Incubation was carried out at 37° for 60 min. At the end of the incubation, the tubes were immersed in boiling water for 60 sec, cooled and centrifuged [2]. The NAD formed was determined enzymically with alcohol dehydrogenase in aliquots of the supernatant [13]. The amounts of the inhibitors contained in the aliquots do not interfere with the quantitative determination of

NAD. The blanks obtained by omission of deamido-NAD and L-glutamine in the reaction mixtures were very low and were substracted from the values given in the tables. Glutamine and its α-*N*-alkyl derivatives liberated only insignificant amounts of ammonia when incubated with the enzyme under the conditions of the experiments, as measured by the method described elsewhere [14].

3. Results and discussion

α-*N*-Alkyl derivatives of glutamine can substitute for glutamine in NAD synthesis, although with less effect (table 1). α-*N*-Dimethyl-L-glutamine is approximately as effective as α-*N*-ethyl-L-glutamine, but the effect of α,α-*N*-methyl-propyl-L-glutamine is less pronounced. Cbz-L-glutamine cannot substitute for glutamine, but inhibited its utilization (table 2). Table 2 also shows that the synthesis is inhibited by Cbz-L-asparagine, Cbz-L-aspartic acid, Cbz-L-alanine, Cbz-L-phenylalanine and Cbz-*S*-benzyl-L-cystein, the inhibition by the latter two compounds being the strongest. The corresponding free amino acids did not inhibit NAD synthesis. The inhibition by Cbz-L-asparagine is competitive with regard to glutamine, whereas the inhibition by Czb-L-phenylalanine is of a mixed type

Table 1
Effect of α-*N*-alkyl derivatives of L-glutamine on rat liver NAD-synthetase.

Glutamine or derivatives	(μmoles)	NAD formed (μmoles)
L-Glutamine	2.5	0.060
L-Glutamine	5.0	0.100
L-Glutamine	10.0	0.125
α-*N*-Ethyl-L-glutamine	20.0	0.096
α-*N*-Propyl-L-glutamine	10.0	0.073
α-*N*-Propyl-L-glutamine	20.0	0.091
α-*N*-Butyl-L-glutamine	20.0	0.077
α-*N*-Amyl-L-glutamine	20.0	0.075
α,α-*N*-Dimethyl-L-glutamine	20.0	0.086
α,α-*N*-Methylpropyl-L-glutamine	20.0	0.055
Carbobenzoxy-L-glutamine	50.0	0.000
NH_4Cl	40.0	0.040

Table 2
Inhibition of rat liver NAD synthetase by carbobenzoxy (Cbz) derivatives of amino acids.

Cbz derivative added*,†	NAD formed (μmoles)	Percent inhibition
None	0.145	–
Cbz-L-glutamine	0.081	44
Cbz-L-asparagine	0.089	37
Cbz-L-aspartic acid	0.078	46
Cbz-L-alanine	0.099	31
Cbz-L-phenylalanine	0.022	85
Cbz-*S*-benzyl-L-cysteine	0.008	95

* 25.0 μmoles of Cbz derivatives were added.
† All the reaction mixtures contained 5.0 μmoles of L-glutamine.

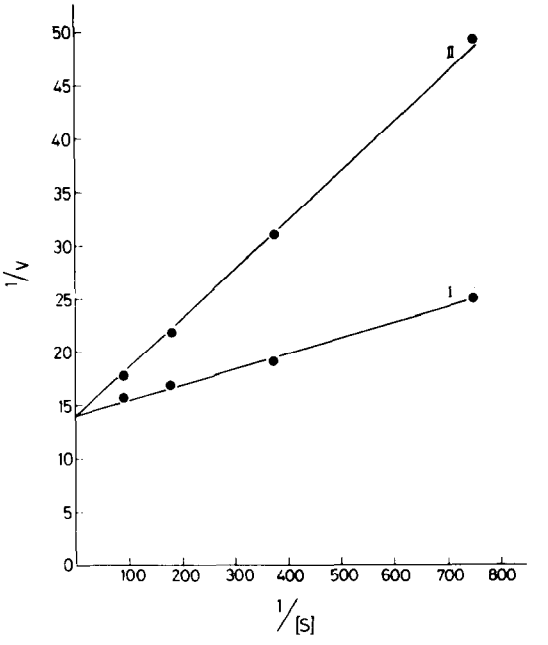

Fig. 1. Inhibition of rat liver NAD synthetase by Cbz-L-asparagine. I) L-glutamine; II) L-glutamine and Cbz-L-asparagine (8×10^{-3} M). [S] is the molar concentration of L-glutamine. Velocity, *V*, is expressed in μmoles NAD formed in 60 min at 37°.

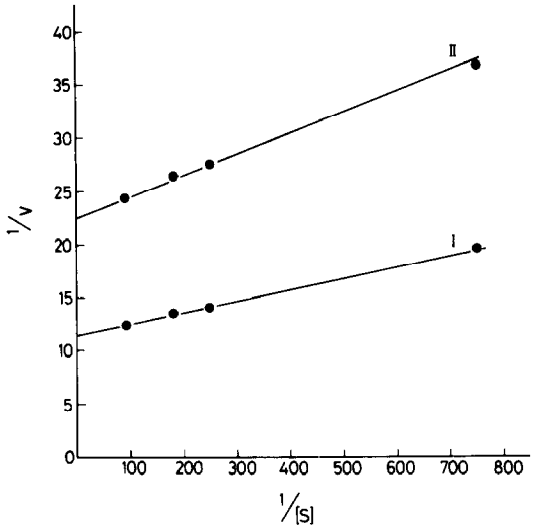

Fig. 2. Inhibition of rat liver NAD synthetase by Cbz-L-phenylalanine. I) L-glutamine; II) L-glutamine and Cbz-L-phenylalanine (5×10^{-3} M). [S] is the molar concentration of L-glutamine. Velocity, *V*, is expressed in μmoles NAD formed in 60 min at 37°.

(figs. 1 and 2). It may be recalled that Cbz-L-phenylalanine and Cbz-*S*-benzyl-L-cysteine strongly inhibit rat liver glutaminase [15], ovine brain and rat liver glutamine synthetase, ovine brain δ-glutamyl transferase [16], as well as rat liver asparaginase [14]. It seems therefore that Cbz derivatives of amino acids with an additional aromatic group inhibit strongly and unspecifically a multitude of enzymes. We might assume, as discussed elsewhere [15], that these inhibitions are caused by hydrophobic interaction between the two aromatic rings of the inhibitor and two suitable sites on the enzyme molecules and that the inhibitions are allosteric in nature. Cbz derivatives of amino acids without an additional aromatic ring (e.g. Cbz-L-asparagine) may also inhibit in an allosteric manner, but since in this case only one aromatic ring binds to the enzyme the inhibition is less pronounced and may be overcome by increasing the glutamine concentration.

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