REGULATION OF NADH AND NADPH METABOLISM IN AZOTOBACTER VINELANDII

H. W. J. VAN DEN BROEK and C. VEEGER
Department of Biochemistry, Agricultural University, Wageningen, The Netherlands

Received 20 September 1968

1. Introduction

From extracts of the nitrogen-fixing bacteria *Azotobacter vinelandii* a reversible NAD^+-dependent NADPH-lipoate reductase and a reversible NAD^+-dependent NADH-lipoate reductase can be separated [1]. The NADPH-lipoate reductase is a complex, which consists of the NADH-specific lipoamide dehydrogenase identical with the one isolated separately and a transhydrogenase. In order to get more information on the role of this transhydrogenase in the regulation of the NADH and NADPH metabolism in *Azotobacter*, kinetic studies were performed with this enzyme.

2. Materials and methods

NAD^+, $NADP^+$, NADPH, lipoic acid and bovine serum albumin were obtained from the Sigma Chemical Co.; NADH, yeast alcohol dehydrogenase and glucose-6-phosphate dehydrogenase from Boehringer. All chemicals used were of analytical grade.

Azotobacter vinelandii (ATCC 478) was grown on a nitrogen-free medium according to Pandit-Hovenkamp [2]. The cells were harvested at the end of the exponential growth phase and stored at −15°C. Lipoamide dehydrogenase from pig heart was prepared by the method of Massey et al. [3], from *Azotobacter* in a similar way. The activity of the pig heart enzyme in the reaction with oxidized lipoic acid was determined according to Massey and Veeger [4]; in the lipoate reductase activity determination of the *Azotobacter* enzyme, the citrate-buffer pH 5.65 was replaced by citrate-phosphate buffer pH 6.35. The preparation of the NADPH-specific lipoate reductase and the transhydrogenase from *Azotobacter* will be published elsewhere [5].

The transhydrogenase activities were estimated by modifications of previous methods [6, 7]. In studies of the $NADP^+$-reduction by NADH the medium consisted of Tris-HCl buffer pH 8.0, 0.1 M; bovine serum albumin, 0.04%; alcohol, 0.15 M; semicarbazide, 4 mM; excess yeast alcohol dehydrogenase; NADH, $NADP^+$, NADPH and $MgCl_2$ as indicated. The NAD^+-reduction by NADPH was carried out in Tris-HCl buffer pH 8.0, 0.1 M; bovine serum albumin, 0.04%; glucose-6-phosphate, 3–6 mM; excess glucose-6-phosphate dehydrogenase; NADPH and NAD^+ as indicated. The reactions were started by the addition of the enzyme and the increase in optical density at 340 nm was measured at 25°C.

3. Results and discussion

When separated, both the NADH-specific lipoamide dehydrogenase and the transhydrogenase do not show any NADPH-lipoate activity, but on recombination the original specificity towards NADPH is recovered. The activity with an equal concentration of NADPH is under the same conditions higher than that with NADH, but is dependent on the ratio transhydrogenase lipoamide dehydrogenase. Upon the addition of the bacterial transhydrogenase to pig heart lipoamide dehydrogenase the rate of the lipoate reduction by NADPH was, in the presence of NAD^+, about twice that with NADH under the same conditions; without NAD^+, or with $NADP^+$ instead of NAD^+,

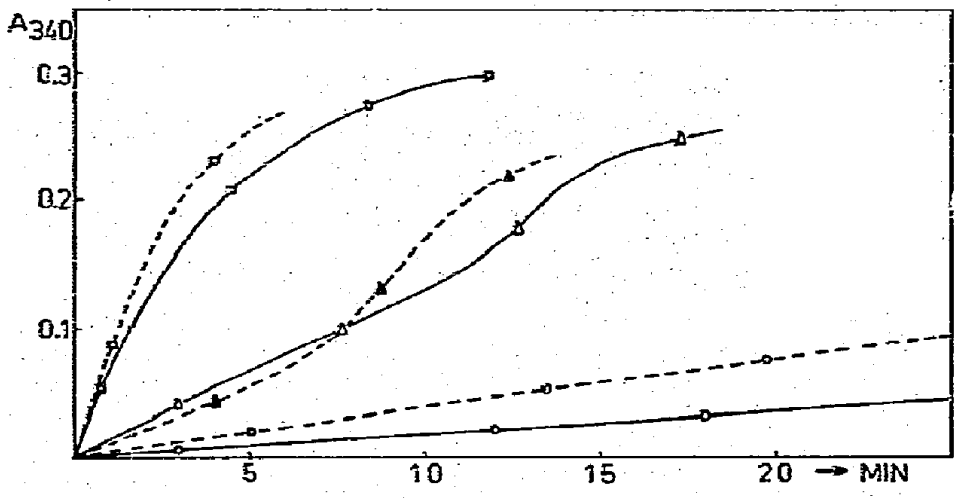

Fig. 1. Time course of the change in optical density at 340 nm as a result of the reduction of $NADP^+$ by NADH in the presence or absence of $MgCl_2$, NADPH and ATP. Reaction mixture as described under materials and methods. [$NADP^+$] = 50 μM; [NADH] = 50 μM. Full and dotted lines are without and with $MgCl_2$ (2 mM) respectively. o—o = no NADPH; □ = 60 μM NADPH; △ = 60 μM NADPH, 16 μM ATP; ▲ = 60 μM NADPH, 800 μM ATP.

this reaction does not proceed. By increasing the ratio transhydrogenase lipoamide dehydrogenase a saturation curve for the NADPH-lipoate activity is obtained, whereas the NADH-lipoate activity remained at a constant level.

The isolated transhydrogenase, which shows a weak polarized flavin fluorescence, is able to reduce 2,6-dichlorophenol-indophenol, $K_3Fe(CN)_6$ and thionicotinamide-NAD^+ with NADH and NADPH; the activities with NADPH, as measured under identical conditions, are about twice that with NADH. 3-Acetylpyridine-NAD^+ is not reduced. Furthermore both the reduction of $NADP^+$ by NADH and that of NAD^+ by NADPH are catalyzed. The rate of reduction of $NADP^+$ by NADH depends on the presence of either $MgCl_2$ or $CaCl_2$; $MgSO_4$ and KCl are much less active. Furthermore NADPH formed during the reaction or added at the beginning has a strongly stimulating effect (fig. 1). $NADP^+$-reduction does not occur in the absence of both $MgCl_2$ and NADPH, unless at very high concentrations of the transhydrogenase. By raising the NADPH/$NADP^+$-ratio the lag period shortens and finally disappears at a ratio of 0.5—1, depending on the [NADH]. In the presence of 2—4 mM $MgCl_2$ the lag period is much shorter, the rate of the reaction is higher and a smaller amount of NADPH is needed to overcome the lag period. The length of the lag period also depends on the concentration of NADH; the higher the [NADH] the higher the [NADPH] needed. A plot of the reaction velocity at variable [NADH] and [NADPH] and constant [$NADP^+$] (fig. 2) shows that NADPH has stimulatory and inhibitory effects, depending on the [NADH]. At increasing [NADH] the activity-NADPH-curve is S-shaped, and shows product inhibition. A similar picture is obtained upon varying [$NADP^+$] and [NADPH] at a constant [NADH]; the S-shaped curves are even more pronounced. The reduction of NAD^+ by NADPH is under the present conditions less complex than the reaction in the reverse direction. The NAD^+-reduction proceeds very quickly. Under identical conditions of donor and acceptor concentrations the rate of NAD^+-reduction is about five to ten times larger than that of the reduction of $NADP^+$.

The rate of NAD^+-reduction depends on the concentrations of both pyridinenucleotides. At increasing [NADPH] the activity-NAD^+-curve changes from a normal saturation curve into an S-shaped curve. It is clear from the results presented that higher concentrations of NAD^+ decrease the affinity of the enzyme for NADPH. NADH has an inhibiting effect on this reaction; $MgCl_2$ has very little influence.

In the presence of a concentration of NADPH high enough to overcome the lag period of the reduction of

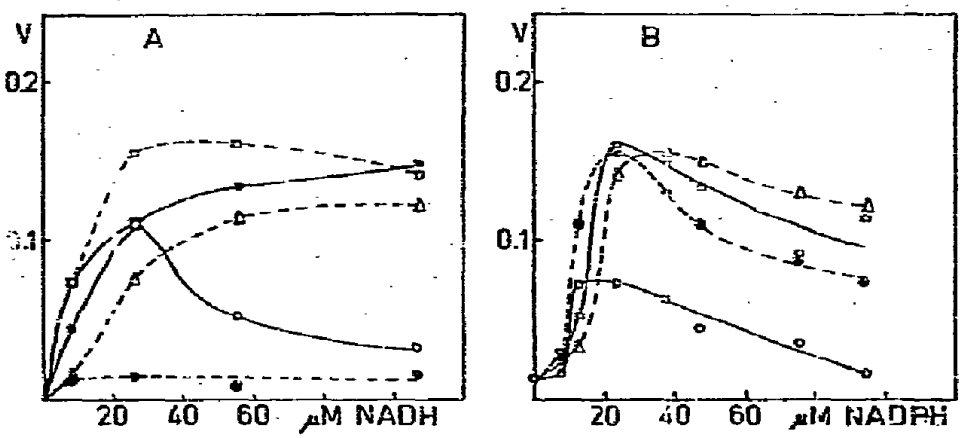

Fig. 2. Effect of NADH and NADPH concentrations on the velocity of the reduction of $NADP^+$ by NADH in the presence of $MgCl_2$. Reaction mixture as described. [$NADP^+$] = 50 μM. The velocity is expressed as the change in optical density at 340 nm for the first three minutes. A. [NADPH]: ●—● = none; ○—○ = 13 μM; □—□ = 25 μM; ■—■ = 50 μM; △—△ = 100 μM. B. [NADH]: ○—○ = 10 μM; ●—● = 25 μM; □—□ = 55 μM; △—△ = 110 μM.

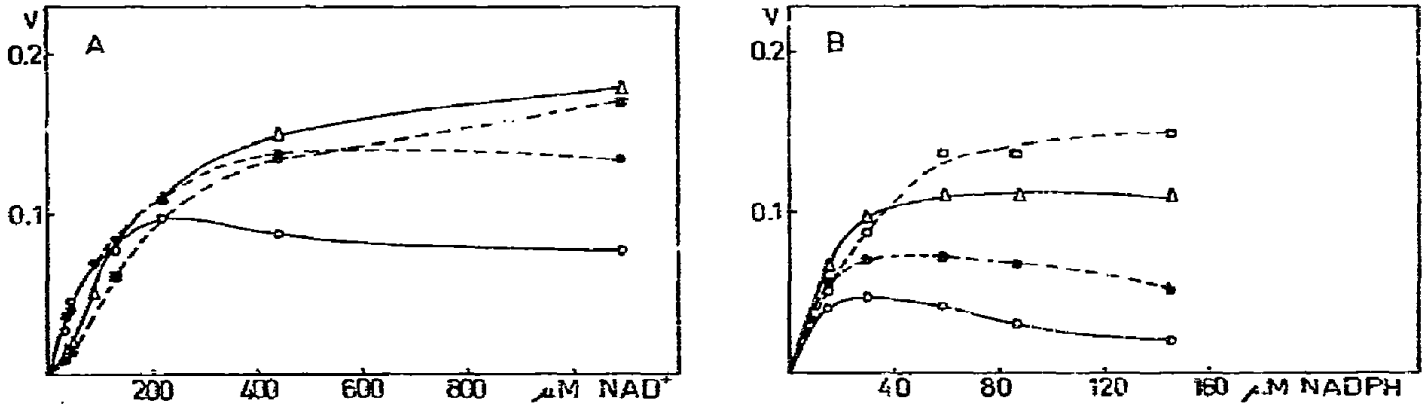

Fig. 3. Effect of NADPH and NAD^+ concentrations on the velocity of the reduction of NAD^+ by NADPH. Reaction mixture as described. The initial velocity is expressed as Δ A/min at 340 nm. A. [NADPH]: ○—○ = 30 μM; ●—● = 60 μM; △—△ = 145 μM; □—□ = 230 μM. B. [NAD^+]: ○—○ = 45 μM; ●—● = 90 μM; △—△ = 220 μM; □—□ = 440 μM.

$NADP^+$ by NADH, addition of ATP induces the lag period again (fig. 1). The ATP-concentration inducing this effect is dependent on the $MgCl_2$ concentration in the medium; in its absence ATP-concentrations as low as 10–40 μM are effective. Furthermore the ATP-inhibition is counteracted by increasing NADPH-concentrations.

The effect of ATP on the NAD^+-reduction by NADPH depends on the concentration of NAD^+ and NADPH. The higher the [NAD^+] the larger the inhibition by ATP. Also here $MgCl_2$ counteracts the ATP-inhibition. GTP has a similar effect on both reactions. Under the same conditions no clear effect was shown with ADP, GDP, 2'AMP, 5'AMP and 3',5' AMP. The catalytic picture shown here with its S-shaped saturation curves indicates a possible allosteric regulation [8] of pyridine nucleotide metabolism by NADPH. Hill-coefficients for NAD^+ of 1.5 and 3–4 for NADPH are obtained, supporting the assumption of a regulatory function of NADPH.

The experiments show the existence of two NADPH binding sites; one catalytic the other regulatory. The regulatory site is negatively affected by ATP and NADH and probably NAD^+ while it is positively affected by $MgCl_2$ and $CaCl_2$. The observation that this transhydrogenase is complexed with lipoamide dehydrogenase makes a direct role of this enzyme in the regulation of transfer of reducing equivalents from pyruvate to either N_2 or O_2 very likely [9].

Acknowledgements

The authors wish to thank Mr. J. Santema for his technical assistance. Part of these investigations was subsidized by the Netherlands Foundation for Chemical Research (S. O. N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z. W. O.).

References

[1] H. W. J. Van den Broek and C. Veeger, Abstr. Fed. European Biochem. Soc. Praha, 1968, p. 17.
[2] H. G. Pandit-Hovenkamp, in: Oxidation and Phosphorylation, eds. R. W. Estabrook and M. E. Pullman (Academic Press, New York, 1966).
[3] V. Massey, Q. U. Gibson and C. Veeger, Biochem. J. 77 (1960) 341.
[4] V. Massey and C. Veeger, Biochim. Biophys. Acta 48 (1961) 33.
[5] H. W. J. Van den Broek and C. Veeger, manuscript in preparation.
[6] S. P. Colowick, N. O. Kaplan, E. F. Neufeld and M. M. Ciotti, J. Biol. Chem. 195 (1952) 95.
[7] N. O. Kaplan, S. P. Colowick and E. F. Neufeld, J. Biol. Chem. 205 (1953) 1.
[8] J. Monod, J. Wyman and J. P. Changeux, J. Mol. Biol. 12 (1965) 88.
[9] L. E. Mortenson, R. C. Valentine and J. E. Carnahan, J. Biol. Chem. 238 (1963) 794.