A FACTOR FOR COUPLING NAD TO HYDROGENASE IN HYDROGENOMONAS EUTROPHA

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Extracts of Clostridium kluyveri were shown by Korkes (1951) to have hydrogenase activity which was coupled with NAD reduction without mediation by artificial carriers. He observed (1955) that an extract from boiled cells added to the enzyme preparation stimulated the rate of NAD reduction. Repaske and Seward (1960) reported that boiled C. kluyveri preparations also stimulated NAD reduction by cell-free hydrogenase from H. eutropha. The active component in the boiled extract was probably FMN, since the flavin containing boiled extract was completely replaced by FMN. Subsequent examination of the C. kluyveri system by Kinsky et al. (1961) showed that, in contrast to the H. eutropha system, C. kluyveri required FAD and at least one other component furnished by the boiled extract. The flavin requirements for NAD reduction by C. kluyveri and H. eutropha extracts differ from the hydrogen dehydrogenase of Hydrogenomonas ruhlandii which has no cofactor requirement (Bone et al., 1963).

Ferredoxin, found in anaerobic microorganisms and in plants, is thought to be a primary electron acceptor during hydrogen activation in anaerobes (Mortenson et al., 1962; Valentine et al., 1962). Ferredoxin is not found in H. eutropha, and clostridial ferredoxin does not replace FMN or augment the rate of NAD reduction by hydrogen with H. eutropha extracts. This communication will present evidence for a factor in H. eutropha extracts which couples hydrogenase with the reduction of NAD in the absence of FMN. Activity is dependent upon ATP.

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Cell-free extracts of autotrophically grown Hydrogenomonas eutropha (Wittenberger and Repaske, 1960) were prepared under anaerobic conditions using a 10 kc Raytheon sonic oscillator. During centrifugation of the extract and all subsequent treatments including storage, the extracts were kept under a hydrogen atmosphere; this was necessary to maintain maximum enzyme activity (Repaske, 1961).

Hydrogenase activity was measured spectrophotometrically by following the rate of NAD or of methylene blue reduction by molecular hydrogen at 340 mμ or 610 mμ, respectively. Controls were run under helium. The rate of NAD reduction with partially purified enzyme preparations was stimulated 10 fold or more by 6.7×10^{-7} M FMN (Repaske, 1961), whereas with crude extracts, the extent of FMN stimulation varied considerably from one preparation to another. Neither FAD nor riboflavin significantly affected NAD reduction when used in place of FMN or in conjunction with it.

When hydrogenase activity was measured by methylene blue reduction, no flavin requirement could be demonstrated; fractions completely dependent upon FMN for NAD reduction reduced methylene blue without added FMN. Comparative hydrogenase rates determined by methylene blue reduction or by NAD reduction with added FMN (mM extinction coefficients for NADH and methylene blue, 6.21 and 35, respectively) differed by less than 20%.

It was found that ATP plus an as yet unidentified factor present in extracts stimulated NAD reduction by hydrogenase without FMN. Results are summarized in Table I. The rate of NAD reduction by the extract was increased 1.5 fold with ATP and 2.3 fold with ATP and Mg^{++}; Mg^{++} alone had no effect. ATP and Mg^{++} added with FMN did not increase the rate from that obtained with FMN alone. These results suggest that there is a common hydrogenase whose maximum activity can be determined by assaying with FMN as cofactor. (As mentioned above, equivalent rates were obtained with methylene blue as electron acceptor.) If there is a common hydrogenase and total hydrogenase activity was determined with FMN, no additional increase in rate would be expected by

ATP even if a second pathway for NAD reduction existed. With limiting FMN, additive stimulation by FMN and ATP should be observed when they are combined. The predicted results were obtained (Table IV).

Table I. Effect of ATP and FMN in coupling hydrogenase and NAD

Additions	ΔO.D.$_{340}$ per min.
None	.136
ATP	.210
Mg^{++}	.124
ATP, Mg^{++}	.308
FMN	.450
FMN, ATP	.466
FMN, ATP, Mg^{++}	.452

The basic reaction mixture contained 0.1 M triethanolamine, pH 7.5; 0.01 M β-mercaptanol and enzyme in the main compartment of a Thunberg type cuvette and 3μmoles of NAD in the cap. Total volume was 3 ml. Other constituents where indicated: 2 μmoles ATP, 3 μmoles $MgCl_2$, 0.002 μmoles FMN. After gassing with hydrogen the cuvettes were preincubated for 5 min. at 35°. NAD was tipped to start the reaction.

ATP stimulation can only be demonstrated if the enzyme and ATP are preincubated under hydrogen for several minutes before NAD is added to initiate the reaction (Table II). Preincubation is not effective unless enzyme and ATP are present together. Adenine, guanine, inosine, uridine and cytosine, mono-, di- and tri- ribonucleotides were compared to determine the specificity of nucleotide stimulation; ATP and ADP were equally active while the other nucleotides were without effect, but adenylic kinase activity in these extracts was not measured. Arsenate, phosphate, 2,4-DNP, Dicumarol, and azide had no effect on ATP stimulation.

The possibility that H. eutropha extracts contained flavokinase which formed FMN with endogenous riboflavin and added ATP was unlikely. Added

riboflavin and ATP preincubated with the extract gave a rate of NAD reduction that was the same as with ATP alone, a rate considerably less than with FMN.

Table II. Effect of preincubation of ATP and enzyme

Conditions	$\Delta O.D._{340}$ per min.
No ATP	.140
ATP with enzyme	.314
ATP with NAD in cap	.134

See Table I for basic reaction mixture and assay conditions. ATP (2 μmoles) was in the main compartment with enzyme or in the cap with NAD as indicated. Preincubation time 5 min.

Crude extract was placed on a Sephadex G-100 column and eluted with 0.005 M triethanolamine, pH 8.0, containing 0.4 M KCl and 0.002 M mercaptoethanol. Two fractions of interest were obtained. One (Fraction H) was eluted early and was enriched for hydrogenase; the other (Fraction T) was well separated and contained the coupling factor (Table III). NAD reduction by hydrogen was determined with each fraction with (1) no cofactor additions, (2) with FMN and (3) with ATP added to the reaction mixture. Hydrogenase was also determined by the methylene blue assay. Hydrogen oxidation and NAD reduction by Fraction H occurred only when FMN was added; a similar rate for hydrogenase activity was found with the methylene blue assay. Fraction T on the other hand had virtually no hydrogenase activity in any of the assays. When the two fractions, H and T, were combined, NAD reduction (with FMN added) was the same as with Fraction H alone, whereas NAD reduction in the presence of ATP was stimulated 10-fold. Increasing the amount of Fraction T did not increase the rate in the ATP reaction; decreasing the amount of Fraction T decreased the rate proportionally.

Table III. NAD coupling factor and hydrogenase fractions from Sephadex G 100 column

	ΔO.D.$_{340}$ per min. Assay*		
	NO ATP or FMN	ATP	FMN
Fraction H	.018	.020	.320
Fraction T	.004	.002	-
Combined H and T.	.086	.212	.300

*Either ATP (2 µmoles) or FMN (0.002 µmoles) was added to the basic reaction mixture. Five minutes preincubation under hydrogen preceded all assays.

Table IV. Comparison of activity with coupling factor and FMN

Additions	ΔO.D.$_{340}$ per min.
None	.100
Fraction T	.108
ATP	.124
Fraction T and ATP	.252
FMN (6.7×10^{-7}M)	.380
FMN (1.7×10^{-8}M)	.138
FMN " and ATP	.180
FMN " and Fraction T	.124
FMN " Fraction T, ATP	.284

The basic reaction mixture (buffer, β-mercaptoethanol $MgCl_2$ and NAD) was supplemented as indicated. Rates were determined after five minutes preincubation.

Table IV presents additional evidence that the stimulation by Fraction T was different from FMN stimulation and therefore was not caused by FMN carried over. The crude cell-free extract was not stimulated significantly by Fraction T or ATP unless these were added together; this rate was less than the maximum obtained with excess FMN (6.7×10^{-7} M). A limiting

concentration of FMN was added to the crude extract to approximate the rate observed with Fraction T alone. ATP plus FMN unlike ATP plus Fraction T gives no real stimulation. Rates obtained with FMN and Fraction T similarly showed no stimulation. However, FMN, Fraction T, and ATP together gave a rate which was additive for the stimulated rate of Fraction T plus ATP and the rate increase due to FMN. It is concluded that Fraction T and FMN produced entirely different responses. The effect of ATP with the former resulted in stimulation of NAD reduction; with the latter, the effect was additive.

These preliminary experiments suggest that there is a factor present in extracts of H. eutropha which couples hydrogenase with NAD. This reaction cannot occur unless a period of preincubation is allowed in order for ATP to react with or modify some component of the system. Whether FMN is a physiological or an artificial coupler in this system remains an open question despite previous consideration of the nucleotide as a natural cofactor for NAD reduction.

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