The Nature of the Cofactor Requirements of the Hydrogenase System from Clostridium kluyveri

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Previous studies in this laboratory showed that the reduction of dihydrophosphopyridine nucleotide with molecular hydrogen, as catalyzed by extracts of Clostridium kluyveri, involves the participation of at least two protein components (1, 2), one of which is ferredoxin (3), and of at least two cofactors obtainable from boiled cell extracts (4). The heat-stable cofactors were separated by column chromatography on Magnesol-Celite (4); one was identified as flavin adenine dinucleotide; the other, referred to as Magnesol cofactor, has not been identified.

This report describes further resolution of the Magnesol cofactor preparation into two subfractions that are independently capable of activating the enzyme system. The active principles in these fractions could not be identified with any of the common nucleotide derivatives, but may be replaced by relatively high concentrations of flavin mononucleotide, adenosine triphosphate, or any of a large number of di- or trinucleotide derivatives. Evidence is presented indicating that these substances serve as activators of the hydrogenase system. A preliminary report of this work has been published (5).

EXPERIMENTAL PROCEDURE

Materials—The sources of the following materials have been given: lactate dehydrogenase, DPN, GSH, Tris, sodium pyruvate, and DEAE-cellulose (3); FAD and Magnesol adsorbent (4). The Clostridium pasteurianum ferredoxin (about 60% pure) was the gift of Dr. J. E. Carnahan of du Pont. The preparations of boiled cell extract (2), and crude C. kluyveri extract, as well as enzyme Fractions H (DEAE-cellulose-treated extract) and F (C. kluyveri ferredoxin) (3), have been published. The Magnesol cofactor preparations used were prepared in 1958 by S. Kinsky according to his method (4) and stored under vacuum at 4°C over CaCl₂. The TT-ribose-P and the purine and pyrimidine bases, nucleosides, and nucleotides were the gift of Dr. R. W. Eschen of this laboratory. Coenzyme A, the acyl-CoA derivatives, and the acylpantetheine derivatives were the gift of Dr. P. R. Vagelos. In addition, we have used FMN, thiamine-PP, d-ribose (Nutritional Biochemicals), TPN (Pabst), and Celite 535 (Johns-Manville).

Resolution of Extract for Cofactor Studies—Crude extracts of C. kluyveri prepared as previously described (3) were resolved of the cofactors required for hydrogenase activity by dialysis. Usually, 10 to 50 ml of crude extract were dialyzed for 45 hours against 100 volumes of distilled water which had previously been thoroughly flushed with hydrogen. The dialysis was performed with stirring at 4°C under an atmosphere of hydrogen. The extract was dialyzed an additional 20 hours under the same conditions but with fresh dialysis medium. The extent of resolution by this method was not highly reproducible and varied with the lot of cells used. A number of other procedures were tried in order to obtain more reproducible resolution. These included treatment of the extracts with charcoal, Florisil, Sephadex G-25, and Dowex 1 (Cl⁻); fractionation with ammonium sulfate, acid, and streptomycin sulfate; aerobic dialysis; and anaerobic dialysis against various buffers, salt concentrations, pH values, urea, reducing agents, FAD, and DPN. None of these procedures gave more consistent results than the dialysis procedure (see also Kinsky et al. (4)), and the latter method was used for the experiments to be discussed.

Activity Measurements—Hydrogenase activity was measured as described in the preceding paper (3) by a manometric assay. Enzyme and cofactor additions were made to a standard mixture which contained, in a final volume of 1 ml, 7 units of lactate dehydrogenase, 5 μmoles of DPN, 40 μmoles of sodium pyruvate, 13.5 μmoles of GSH, and, unless otherwise indicated, 60 μmoles of Tris-HCl, pH 7.8; 0.2 ml of 20% KOH was present in the center well. The flasks were flushed with hydrogen for 5 min and incubated for an additional 25 min. The reactions, started by the addition of lactate dehydrogenase, DPN, and pyruvate from the side arm, were incubated at 30°C.

Analytical Procedures—Protein concentrations were estimated by the method of Lowry et al. (6). Inorganic orthophosphate was determined by the procedure of Tausskey and Shorr (7). Acid-labile phosphate was determined by the difference in total inorganic orthophosphate before and after hydrolysis for 7 min at 100°C in 1 N HCl.

Adenosine triphosphate and ADP were determined by the methods of Kornberg (8) and Kornberg and Pricer (9). These methods measure the reduction of TPN by glucose-6-P in the presence of glucose-6-P dehydrogenase. The glucose 6-P was in turn generated from glucose, ATP, and hexokinase. ADP was determined by conversion to ATP by myokinase.
Diphosphopyridine nucleotide was measured with the use of ethanol and a DPN-specific alcohol dehydrogenase (10). Triphosphopyridine nucleotide was measured by its reduction to TPNH in the presence of glucose-6-P and glucose 6-phosphate dehydrogenase.

RESULTS

In confirmation of previous results obtained with other assay procedures (4), the data of Table I show that dialysis of crude extracts results in almost complete loss of DPN-linked hydrogenase activity and that the activity can be partially restored by addition of a mixture of FAD and Magnesol cofactor, but not by either one alone. The data show also that added ferredoxin does not replace either of the cofactors; moreover, it causes little stimulation of a dialyzed preparation supplemented with both cofactors. The latter observations are particularly noteworthy since they contraindicate the validity of earlier assumptions (11) that ferredoxin was a component of the cofactor preparations used by Korkes (1, 12) to reactivate dialyzed enzyme preparations. The nonidentity of ferredoxin and the Magnesol cofactor is further indicated by the observations that ferredoxin is heat-labile and Magnesol cofactor is heat-stable; ferredoxin has an absorption peak at 396 nm (Fig. 4 of Reference 3) and Magnesol cofactor has no such peak; Magnesol cofactor is dialyzable and ferredoxin is not; and finally, ferredoxin has a high affinity for DEAE-cellulose and Magnesol cofactor has a relatively low affinity (see below).

Failure of added cofactors to stimulate the activity of crude undialyzed extracts indicates that these extracts contain saturating quantities of the cofactors. The slight inhibition obtained with addition of Magnesol cofactor preparations is due to their contamination with inhibitory substances. This conclusion is consistent with the subsequent observation that chromatography of Magnesol preparations on carboxymethyl cellulose results in a consistent with the subsequent observation that chromatography

**Table I**

*Multiple requirements for hydrogenase activity*

<table>
<thead>
<tr>
<th>Additions</th>
<th>Undialyzed extract</th>
<th>Dialyzed extract</th>
<th>DEAE-cellulose-treated extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen uptake</td>
<td>µl/mg protein/hr</td>
<td>µl/mg protein/hr</td>
<td>µl/mg protein/hr</td>
</tr>
<tr>
<td>None</td>
<td>12.2</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Magnesol cofactor</td>
<td>9.2</td>
<td>2.0</td>
<td>1.2</td>
</tr>
<tr>
<td>FAD</td>
<td>12.7</td>
<td>1.4</td>
<td>0.6</td>
</tr>
<tr>
<td>Magnesol cofactor + FAD</td>
<td>9.9</td>
<td>6.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Ferredoxin</td>
<td>16.3</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>Ferredoxin + Magnesol cofactor</td>
<td>12.8</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Ferredoxin + FAD</td>
<td>17.9</td>
<td>2.2</td>
<td>3.9</td>
</tr>
<tr>
<td>Ferredoxin + Magnesol cofactor + FAD</td>
<td>13.1</td>
<td>7.9</td>
<td>1.9</td>
</tr>
</tbody>
</table>

To the standard reaction mixtures were added as indicated: FAD, 18 mmoles; Magnesol cofactor, 8 mg; *C. pasteurianum* ferredoxin, 0.25 mg; undialyzed extract, 5.5 mg (protein); dialyzed extract, 6.0 mg (protein); and DEAE-cellulose-treated extract, 4.9 mg (protein). These data are from 3 days of experiments. On successive days, the effects of various cofactors were examined with undialyzed, dialyzed, and DEAE-cellulose-treated extract, respectively. DEAE-cellulose-treated extract is equivalent to enzyme Fraction H, i.e. freed of ferredoxin.

**Table II**

*Interrelations of various cofactor and protein fractions*

To the standard reaction mixtures were added 19 mmoles of FAD and 8 mg of Magnesol cofactor wherever indicated. The levels (milligrams of protein) of extract used were in the case of the undialyzed extract experiments: 15 mg of crude extract, 13.6 mg of Fraction H, and 1.4 mg of Fraction F corresponding to a total of 15 mg when H and F were added together. Dialyzed extracts were added at the following levels: 8.5 mg of crude extract, 8.1 mg of Fraction H, and 0.4 mg of Fraction F corresponding to a total of 8.5 mg when H and F were tested in combination. This table summarizes the data from six independent Warburg experiments, with the use of the same reagents in each case. Only the enzyme fractions being tested for their response to FAD and Magnesol cofactor were changed.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Hydrogen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undialyzed extract</td>
</tr>
<tr>
<td></td>
<td>µl/mg protein/hr</td>
</tr>
<tr>
<td>None</td>
<td>147</td>
</tr>
<tr>
<td>FAD</td>
<td>158</td>
</tr>
<tr>
<td>Magnesol cofactor</td>
<td>134</td>
</tr>
<tr>
<td>FAD + Magnesol cofactor</td>
<td>140</td>
</tr>
</tbody>
</table>

* The data are from an experiment in which Fractions H and F were prepared by DEAE-cellulose chromatography of a dialyzed crude extract. Comparable results were obtained when Fraction H + F prepared by chromatography of undialyzed crude extracts was subsequently dialyzed.

20% increase in total activity. Added ferredoxin consistently stimulated the crude undialyzed extract. The percentage of stimulation was greater when ferredoxin was added to a relatively small amount of crude extract. This effect is consistent with previous studies showing that ferredoxin (3) is one of two enzymes (1, 2) limiting in the over-all reaction system.

As was noted in the previous report (3), chromatography on DEAE-cellulose resolves the crude extracts into two protein fractions, H and F, containing the hydrogenase and ferredoxin, respectively. The last column of Table I shows that DEAE-cellulose-treated extract (Fraction H) has little activity by itself or when supplemented with either or both FAD or Magnesol cofactor, but it is activated by additions of pure ferredoxin. The interrelations between the various cofactor and protein fractions are further indicated by the data of Table II. In the absence of any added enzyme, FAD and Magnesol cofactor, alone or in combination, have no activity of themselves. Crude, undialyzed extracts require no additions for activity and are not stimulated by additions of FAD, Magnesol cofactor, or a combination of the two. Fractions H and F obtained by DEAE-cellulose chromatography of crude, undialyzed extracts are inactive when tested independently and neither responds to additions of Magnesol cofactor and FAD. However, a mixture of Fractions H and F obtained from undialyzed extracts is very active and is not stimulated by the cofactor additions. Thus, whereas DEAE-cellulose chromatography permits separation of Fractions H and F, it does not result in resolution with respect to the heat-stable cofactors.

On dialysis, the activity of crude extracts is lost. A combina-

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uptake is proportional to the square of the FAD concentration.

Suggests involvement of more than 1 mole of FAD in the reaction. Under these conditions, the reaction appears to be bimolecular with respect to FAD since the rate of hydrogen uptake is proportional to the square of the FAD concentration.

Magnesol cofactor is involved. As pointed out by Atkinson and Walton (13), this interpretation is but one of several possibilities. The kinetics of regulatory enzymes is often so complex that they do not lend themselves to conventional analyses.

Nature of Magnesol Cofactor Effect — A possible role of the Magnesol cofactor is indicated by the data of Fig. 2 which show the influence of FAD concentration on hydrogen uptake in the presence and absence of Magnesol cofactor. It is evident that a marked stimulation by Magnesol cofactor is demonstrable only at relatively low FAD concentrations (0 to 100 nM) and could not be recovered by elution with high concentration of salt.

The diffuse distribution of activity during chromatography suggested the presence of more than one active component in the cofactor preparations. This possibility is supported by the data of Fig. 1, showing that chromatography of boiled cell extracts on DEAE-cellulose columns, with a shallow salt gradient for elution, resulted in the separation of two major peaks of activity. About 13% of the original activity was recovered.

The second peak of activity (Fract. 8 and 9) cochromatographed with FAD and was freed of this contaminant by passage over a Magnesol-Celite column (4) before assay. These data confirm the existence of at least two active components either of which in addition to FAD, will cause activation of the hydrogenase system. A combination of limiting quantities of the two active components gave roughly additive results. Although the identity of these compounds remains obscure, they appear to be weakly anionic and at the concentrations studied show no absorption at wave lengths greater than 400 m. The absorption of both fractions at 450 m was less than 1% that observed at 260 m.

Nature of Magnesol Cofactor Effect — As noted earlier, there is an influence of FAD and Magnesol cofactor partially restores the activity (see also Table I). Dialyzed Fractions H and F are inactive when tested independently with or without the cofactor additions. However, mixtures of dialyzed Fractions H and F, although relatively inactive in the absence of added cofactors, are active when FAD and Magnesol cofactor are both added. These data show that little enzyme activity is demonstrable in the absence of any one of the four components, Fraction H, Fraction F (ferredoxin), FAD, and the Magnesol cofactor.

Fractionation of Magnesol Cofactor Preparation — Efforts to achieve a further purification of the Magnesol cofactor by paper and ion exchange chromatography were not encouraging. Paper chromatography in a variety of solvent systems resulted in low recoveries of activity. After chromatography, the cofactor activity was not localized in a discrete area but was generally diffusely distributed throughout the chromatogram.

A DEAE-cellulose chromatography of boiled cell extract. A DEAE-cellulose column (3 X 18 cm) was prepared and washed with water, 2% ammonium formate, and again by water. To this column were added 38 ml of boiled cell extract. Material not adsorbing to the column was flushed with 100 ml of water and collected as Fraction F. The column was eluted with a 1000 ml linear ammonium formate gradient (0 to 0.34 M), collecting about 100-ml fractions (Fract. 1 to 14). Finally, the column was stripped with 100 ml of 2% ammonium formate followed by 100 ml of water. This constituted Fraction 15. Each of the fractions was lyophilized for 4 to 6 days to remove salt and water. Each of the residues was then dissolved with water to a final volume of 5 ml.

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These effects are more readily observed when double reciprocal
obligatory requirement for FAD. Fig. 2A (Curves 1 and 2) shows that FMN cannot be substituted for FAD either in the presence or absence of added Magnesol cofactor. However, a high concentration of FMN will replace Magnesol cofactor (Fig. 4A) in the stimulation of hydrogen uptake at low FAD concentrations.

**Fig. 2.** The cofactor requirements for hydrogen consumption. To the standard reaction mixtures were added dialyzed *C. kluyveri* extract equivalent to 6.6 mg of protein, and as indicated on the abscissa, FMN (Curves 1 and 2) or FAD (Curves 3 and 4). The data in Curves 2 and 4 are from reaction mixtures containing 10 mg of Magnesol cofactor. This figure illustrates data from 2 successive days. The same reagents and enzyme were used on each day except that the response to FAD was measured on the 1st day and the response to FMN was measured on the next. In Inset B, the rate of hydrogen uptake (microliters per hour) is plotted against the square of the FAD concentration. The data correspond to that plotted in Curve 3, Part A, for the range of FAD concentrations from 0 to 54 µM.

**Fig. 3.** Lineweaver-Burk coordinates. The data for the FAD concentration curves from Fig. 2 are replotted as (A), 1/V against 1/S, and (B) 1/V against 1/S', in which V = hydrogen consumption in microliters per hour minus control (no FAD present) rate, and S = FAD concentration (micromolar). •---•, FAD alone; O---O, FAD plus Magnesol cofactor.

**Fig. 4.** Alterations in the reaction kinetics of FAD elicited by FMN and ATP. A, effect of FMN on FAD kinetics. To the standard reaction mixtures were added dialyzed *C. kluyveri* extract equivalent to 6.2 mg of protein, and FAD, FMN, and Magnesol cofactor as indicated. The FAD concentration curves were run with the following additions: Curve 1, none; Curve 2, 19 mmoles of FMN; Curve 3, 97 mmoles of FMN; Curve 4, 194 mmoles of FMN; or Curve 5, 8 mg of Magnesol cofactor. This figure illustrates data obtained over a 4 day period. The same reagents and enzyme were used in each case. B, effect of ATP on FAD kinetics. To the standard reaction mixtures were added dialyzed *C. kluyveri* extract equivalent to 6.3 mg of protein and FAD as indicated. The FAD concentration curves were run with the following additions: O, none; Δ, 8 mg of Magnesol cofactor; or •, 1 µmole of ATP.

Thus, the parabolic response to increasing FAD concentration noted in the absence of FMN or Magnesol cofactor (Fig. 4A, Curve 1) is less prominent in the presence of 19 µm FMN (Curve 2) and is completely absent at 97 µm FMN (Curve 3) or in the presence of 8 mg of Magnesol cofactor (Curve 5). With higher concentrations of FMN (Curve 4), little further effect is observed. The greater maximal activity obtained with FMN as compared to Magnesol cofactor is attributed to the presence of inhibitors in the latter preparations. In view of the activating effect of FMN, the specificity of this effect was examined. Data presented in Fig. 4B show that ATP at high concentrations (1.0 mM) will also replace the Magnesol cofactor. Other data summarized in Table III show that a number of compounds at 1 mM concentrations are able to activate the hydrogenase system. These included all the nucleoside diphosphates and triphosphates. From Lineweaver-Burk plots, the apparent $K_m$ values for ATP and ADP were estimated at approximately 0.3 mM and 0.4 mM, respectively. Magnesium had no effect or was inhibitory when assayed alone or with ATP or Magnesol cofactor. It is significant that addition of Magnesol cofactor, AMP, or ADP to mixtures containing 1 mM ATP did not appreciably stimulate the reaction. Even at 1 mM concentrations, the nucleoside monophosphates were relatively inactive as were orthophosphate, pyrophosphate, PP-ribose-P, ribose, and thiamine pyrophos-
The discovery that ferredoxins are required for the hydrogenase activities of *C. pasteurianum* (14) and *Micrococcus lactilyticus* (15), and the further observation that these are moderately heat-stable (16), led to the speculation (11) that ferredoxin is identical with a cofactor shown by Korkes (1, 12) to be needed for the hydrogenase system of *C. kluyveri*. Although subsequent work established a role of ferredoxin in the *C. kluyveri* hydrogenase system (3), data presented in this paper show that it is not identical with heat-stable cofactor.

The observation that the rate of hydrogen uptake is a linear function of the square of the FAD concentration in the absence of Magnesol cofactor, but is a linear function of FAD concentration when cofactor is present, is analogous to situations recently encountered with a number of other enzyme systems. Similar kinetic relationships have been observed for deoxythymidine kinase (17), DPN isocitrate dehydrogenase (18, 19), phosphofructokinase (20, 21), 2′-deoxyribo-4-aminopyrimidone 2′,5′-phosphate aminohydrolase (22), and others. Although other interpretations are possible (13, 23), these results are generally interpreted to indicate the presence of two sites on these enzymes that are capable of binding the respective substrates. One site, according to this hypothesis, is a catalytic site whereas the second site is a regulatory (allosteric) site. It is presumed that binding of substrate at the latter site induces conformational changes in the protein that result in an increased affinity of the enzyme for its substrate at the catalytic site. This interpretation is supported by the fact that in each instance addition of specific substances (activators), which are not structurally related to the various substrates, results (a) in typical Michaelis-Menten saturation kinetics that are not bimolecular with respect to the substrate and (b) in an increased affinity of the enzyme for its substrate. It is assumed that the activator molecules can replace the activator function of the substrate when they are tested individually. On the other hand, it is possible that activation by the Magnesol cofactor preparation is due to the additive effects of numerous minor components which individually would cause only slight activation in the concentrations used. Certain restrictions can be imposed on this interpretation. Nucleoside di- and triphosphates cannot collectively contribute a significant amount of activity since the total amount of orthophosphate released by 7 min hydrolysis at 100° in 1 N HCl is only 0.00 µmole/4 mg of Magnesol cofactor preparation. Also, the nonidentity of nucleotide pyrophosphate derivatives with the active substances was previously indicated by the stability of cofactor activity to acid hydrolysis (4). Moreover, the total nucleotide plus nucleoside concentration of a solution containing 4 mg of Magnesol cofactor is roughly estimated to be 1 mm as determined by the absorbance of 12.0 for the solution at 260 mµ. It is evident that not all of this ultraviolet-absorbing material is due to the active compounds. Fractions obtained by DEAE-cellulose chromatography of boiled cell extract (Fig. 1) and diluted to have an activity (per unit volume) equal to the Magnesol cofactor preparations showed far less absorption at 260 mµ: Fraction 3 (0.08), Fraction 4 (1.61), Fraction 8 (1.53), and Fraction 9 (0.075). Therefore, the factors present in the Magnesol cofactor preparations must have a low extinction coefficient, be effective at low concentrations, or both.

**DISCUSSION**

The discovery that ferredoxins are required for the hydrogenase activities of *C. pasteurianum* (14) and *Micrococcus lactilyticus* (15), and the further observation that these are moderately heat-stable (16), led to the speculation (11) that ferredoxin is identical with a cofactor shown by Korkes (1, 12) to be needed for the hydrogenase system of *C. kluyveri*. Although subsequent work established a role of ferredoxin in the *C. kluyveri* hydrogenase system (3), data presented in this paper show that it is not identical with heat-stable cofactor.

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Considered in the light of this interpretation, the hydrogenase
of C. kluyveri would possess two binding sites for FAD. One of these sites would represent the substrate or catalytic site at which FAD would serve an electron acceptor function, whereas binding of FAD at the second, regulator (allosteric), site would result only in conformational changes of the protein.

Activation by Magnesol cofactor, FMN, or any of the other numerous nucleotides examined would therefore, be due to the binding of these, rather than FAD, at the regulatory site; conformational changes thereby induced could account for the increased affinity of the enzyme for FAD at the catalytic site. According to this interpretation, the bimolecular kinetics observed with respect to FAD concentration may have no special biological significance but may merely reflect the incidental ability of FAD to react at the highly nonspecific regulator binding site.\(^4\) Also, according to this interpretation, the Magnesol cofactor would not function as an electron carrier as was previously proposed \(^4\) but rather as an allosteric activator. This view is supported by the discovery that high concentrations of compounds not likely to act as electron carriers, e.g. ATP and ADP, will satisfy completely the cofactor requirement. This consideration appears to exclude also the possibility that the bimolecular kinetics of FAD observed in the absence of cofactor is due to the existence of two separate FAD-binding sites both of which are concerned with electron transport functions.

Whereas the above interpretations are consistent with the experimental observations, they are regarded as tentative and serve as the basis of further study. The limitations of evaluating kinetic data derived from studies with regulatory enzymes have been duly emphasized by Atkinson et al. \((13, 23)\), whose studies on the regulation of yeast phosphofructokinase activity illustrate the difficulties in arriving at unambiguous conclusions from kinetic data such as those reported here.

Allosteric control of the DPN-linked hydrogenase activity could form an effective basis of cellular regulation, since it could determine the relative contribution of pyridine-linked biosynthetic reactions and purely oxidative reactions in which molecular hydrogen is involved as the ultimate reduced product. The importance of such cellular regulation has been recently reviewed \((24)\).

From the data obtained by Peel \((25)\), it can be calculated that the total intracellular concentration of FAD in C. kluyveri is about \(5 \times 10^{-4} \text{M}\). This is considerably in excess of that needed \((2 \times 10^{-4} \text{M})\) to saturate both FAD-binding sites of the hydrogenase. Although this raises questions concerning the significance of allosteric activation by the Magnesol cofactor, such concern may be unreasonable since most of the FAD could be bound to protein constituents and may therefore be unavailable for allosteric control of the hydrogenase.

Repaske and Seward \((26)\) suggested that FMN might be the active principle of the Magnesol cofactor preparations needed for the C. kluyveri hydrogenase system, since low concentrations of FMN \((10^{-7} \text{M})\) can replace boiled cell extracts of C. kluyveri as a source of cofactor needed for the reduction of DPN by molecular hydrogen catalyzed by enzyme preparations of \textit{Hydrogenomonas eutrophu}. This possibility is discounted by the data of Kinsky, Stadtman, and Maclay \((1)\) showing that the concentration of FMN in the Magnesol cofactor preparation is very much less than that required to cause appreciable activation of the C. kluyveri enzyme. Nevertheless, as shown by the present report, high concentrations of FMN \((10^{-4} \text{M})\) will satisfy the Magnesol cofactor requirement; it cannot, however, replace the requirement for FAD. The effect of FMN is therefore no different from that exerted by ATP and other substances incapable of serving as electron carriers. All these substances cause a decrease in the apparent \(K_a\) for FAD at the catalytic site and are presumably allosteric effectors. Accordingly, it appears likely that the effect of FMN in the C. kluyveri hydrogenase system is quite different from that observed with the II. eutrophu enzyme where an electron carrier function is indicated.

**Summary**

Cofactor requirements for the reduction of diphosphopyridine nucleotide with molecular hydrogen by enzyme preparations of \textit{Clostridium kluyveri} were reinvestigated.

The results confirmed previous conclusions that flavin adenine dinucleotide and a factor in boiled extracts ("Magnesol cofactor"), separable from FAD by chromatography on Magnesol, were involved. Whereas the FAD requirement is absolute at all concentrations of reactants tested, activation by the Magnesol cofactor is observed only at relatively low concentrations of FAD. In the absence of Magnesol cofactor, hydrogen-linked DPN reduction is a bimolecular function of the FAD concentration. However, when Magnesol cofactor is present, DPN reduction follows normal Michaelis-Menten saturation kinetics with respect to FAD, and the affinity of the enzyme for FAD is much higher than when Magnesol cofactor is absent. Similar activation of the hydrogenase system is obtained when the Magnesol cofactor is replaced by high concentrations of flavin mononucleotide, adenosine triphosphate, adenosine diphosphate, or any of a large number of other nucleoside di- or triphosphates. The results suggest the presence of two binding sites on the hydrogenase, namely, a catalytic site capable of binding FAD and an allosteric site capable of binding factors present in the Magnesol cofactor preparation and also nucleotides including FAD, flavin mononucleotide, adenosine triphosphate, and adenosine diphosphate when these are present at high concentrations.

The Magnesol preparation was resolved by diethylaminoethyl cellulose chromatography into two active fractions, neither of which contained a sufficient concentration of any of the common nucleotides to account for its activity.

Previous suggestions that the Magnesol cofactor activity is due to flavin mononucleotide or ferredoxin were shown to be incorrect.

**References**

The Nature of the Cofactor Requirements of the Hydrogenase System from Clostridium kluyveri
Walter W. Fredricks and E. R. Stadtman