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IDENTIFICATION AND QUANTITATIVE DETERMINATION OF THE FLAVIN COMPONENT OF SOLUBLE HYDROGENASE FROM ALCALIGENES EUTROPHUS

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SUMMARY

The flavin component of soluble hydrogenase (hydrogen:NAD^+ oxidoreductase, EC 1.12.1.2) from Alcaligenes eutrophus was identified as FMN by thin layer chromatography in two solvent systems and by binding studies with apoflavodoxin from Megasphaera elsdenii. The flavin of hydrogenase reacted rapidly with apoflavodoxin with almost complete quenching of the fluorescence at 525 nm. Quantitative determination of FMN was performed by fluorimetric titration with a standardized solution of apoflavodoxin. From the determined FMN content of different enzyme preparations and from the percentage of stimulation of hydrogenase activity by exogenous FMN it is concluded that hydrogenase contains 2 FMN per molecule.

INTRODUCTION

In the last few years, purified hydrogenases have been characterized from Clostridium pasteurianum (1-3), Desulfovibrio vulgaris (4-7), Chromatium (8,9), Thiocapsa roseopersicina (10), Rhodospirillum rubrum (11) and Alcaligenes eutrophus (12). All of them were identified as an iron-sulfur protein, however, the soluble hydrogenase (hydrogen:NAD^+ oxidoreductase, EC 1.12.1.2) from A. eutrophus was the first and until now the only one described to contain a flavin component as additional enzyme-bound electron carrier (12). Only Kakuno et al. (9) recently presented the first indication for the possibility that the hydrogenase from Chromatium is associated with a flavopeptide or a flavoprotein component. On

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isoelectric focussing, they observed a flavin-containing fraction detached from the enzyme band. For soluble hydrogenase from A. eutrophus, the existence of the flavin was indicated by little acceptor specificity and diaphorase activity of the enzyme and finally proved by a strong fluorescence at 526 nm and a flavin typical absorption profile of the isolated chromophore with peaks at 375 and 447 nm. In this report we describe the identification of the flavin component as FMN and present evidence that one mol of hydrogenase contains two mol of FMN.

MATERIALS AND METHODS

Chemicals. The chemicals used were: from Boehringer, NAD, FMN; from Serva, riboflavin; from Sigma Chemicals Co., FAD. Samples of snake venom (Naja naja), apoflavodoxin and highly purified FMN were gifts from Dr. S.G. Mayhew. All other chemicals were obtained from Merck.

Enzyme purification. Soluble hydrogenase was purified from autotrophically grown cells of Alcaligenes eutrophus (Hydrogenomonas H16) as described previously (12).

The protein content of pure enzyme preparations was determined by the Lowry method (13) as well as by the biuret method (14). The values obtained from both methods were approximately the same.

Assay of hydrogenase. Hydrogenase activity was measured spectrophotometrically by following the reduction of NAD (12).

Flavin determination. Trichloroacetic acid extraction of flavin was conducted according to Mayhew and Massey (15).

Thin layer chromatography of flavins was carried out with the use of TLC cellulose plates from Merck, Darmstadt. n-Butanol-acetic acid-water (4 : 3 : 3) and 5% Na_2HPO_4 (in water) were used as solvent systems. 40 µM flavin solutions were prepared and 5 µl of each submitted to chromatography. Flavins were detected by their fluorescence in ultraviolet light.

The flavin of hydrogenase was determined quantitatively by fluorimetric titration with apoflavodoxin from Megasphaera elsdenii (Peptostreptococcus elsdenii) a new sensitive method, recently described by Wassink and Mayhew (16). Fluorescence was measured in a Hitachi fluorescence spectrophotometer 204 at room temperature and at an excitation wavelength of 445 nm and an emission wavelength at 525 nm. The apoflavodoxin, which binds FMN specifically with quenching of the flavin fluorescence (17), was dissolved in 10 mM potassium phosphate buffer (pH 7.0) including 1 mM EDTA to give a final concentration of approximately 20 µM. This solution was then standardized by adding increments of 3 µl to the standard assay mixture (0.4 ml), which contained in a cylindrical fluorescence microcuvette: 1 µM FMN, 10 mM sodium acetate buffer (pH 6) and 200 mM NaCl. The additions of apoflavodoxin were continued until the fluorescence reached a minimum. In a second titration the standardized solution of apoflavodoxin was used to determine the flavin extracted from hydrogenase.

RESULTS AND DISCUSSION

Identification of the flavin component of hydrogenase

Flavin solution prepared from soluble hydrogenase of Alcaligenes eutrophus (Hydrogenomonas H16) was submitted to thin layer chromatography. The chromatograms showed single fluorescent spots with R_f-values nearly identical (Na_2HPO_4-system) or exactly identical (n-butanol-acetic acid-water-system) with those of FMN (Table 1).

Conclusive evidence, that the flavin component of hydrogenase is FMN, was obtained from binding studies with apoflavodoxin from Megasphaera elsdenii (Peptostreptococcus elsdenii). Mayhew (17) has shown that one molecule of apoflavodoxin binds one molecule of FMN to give a complex identical with the native flavodoxin. The binding of the FMN is accompanied with almost complete quenching of the flavin fluorescence. In addition, apoflavodoxin is very specific for FMN and has no detectable reaction with other flavins. In accordance with these observations we have found that the fluorescence of the flavin isolated from different hydrogenase preparations was quenched 97-99% by adding a slight excess of apoflavodoxin. Treatment of the flavin solutions either in the presence or absence of apoflavodoxin with snake venom (Naja naja) as a source of phosphodiesterase, which is known to hydrolyze FAD to FMN, did not change fluorescence itensity. This was consistent with the results of control experiments, where Naja naja venom had no effect on FMN, but caused an 8.5-fold increase of fluorescence during hydrolysis of FAD.

The pH-dependent fluorescence of hydrogenase flavin showed an optimum curve which is typical for FMN (Fig. 1). The fluorescence was nearly constant over a wide pH range between 4 and 9. With FAD a pronounced optimum around pH 3 and only a weak fluorescence at pH 7 would be expected (18).

TABLE 1

THIN LAYER CHROMATOGRAPHY OF HYDROGENASE FLAVIN

Solvent system 1 was n-butanol-acetic acid-water (4 : 3 : 3) and solvent system 2 was 5% Na_2HPO_4 (in water). In this experiment hydrogenase flavin was released from the protein by heat treatment in darkness in a sealed tube at 100° C for 10 min.

Flavin	R_f values	
	solvent 1	solvent 2
Riboflavin	0.45	0.17
FAD	0.16	0.31
FMN	0.30	0.40
Hydrogenase flavin	0.30	0.39

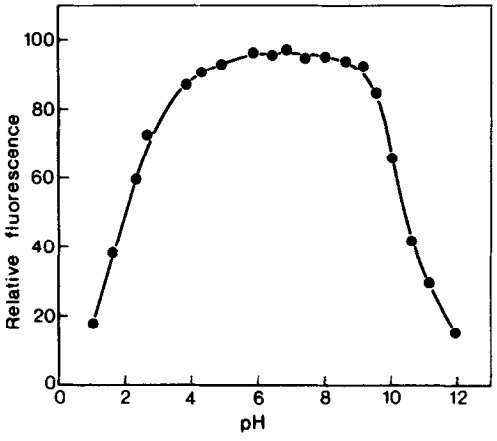

Fig. 1. pH dependence of fluorescence of the flavin component from hydrogenase.

Quantitative determination of FMN

Based on the finding, that the flavin of hydrogenase is FMN and reacts rapidly with apoflavodoxin from M. elsdenii, the fluorimetric titration with this protein (16) turned out to be an excellent method to determine the FMN quantitatively. As we were able to confirm that commercial preparations of FMN contain about 30%

fluorescent impurities (16), for accurate determination of apoflavodoxin we used a preparation of FMN from Mayhew and Strating, which was highly purified by affinity chromatography with apoflavodoxin as the immobilized ligand (19). The titration curves of Fig. 2 demonstrate that the fluorescence decrease of both the standard FMN and the FMN of hydrogenase, were linear dependent on the amount of apoflavodoxin added to the flavin solutions. The concentration of hydrogenase FMN was calculated from the breakpoint in the curve, where the fluorescence reaches a minimum near zero, and where the FMN concentration is equal to the apoflavodoxin concentration. As presented in Table 2 the FMN content of different enzyme preparations varied from 1.1 to 1.4 mol per mol of hydrogenase. By testing enzyme activity we observed that addition of exogenous FMN (1 μM) to the reaction mixture resulted in a 50 and 58% activation of hydrogenase in two preparations and even a 80% activation in a preparation of lower specific activity. The final NAD reduction rate of activated hydrogenase was nearly the same in all preparations and amounted to 65-71 units/mg protein. This is in agreement with previous data reported by Schneider and Schlegel (12), who purified hydrogenase to a specific activity of 54.5 units/mg protein. This preparation was found to be stimulated 25% by FMN to give a final activity of about 68 units/mg protein. It should also be mentioned that FAD and riboflavin were not able to substitute for FMN and had no effect on enzyme activity. The observation that FMN is a coenzyme of hydrogenase and stimulates activity of the purified enzyme, also confirms earlier reports, in which hydrogenase in extract preparations of A. eutrophus (Hydrogenomonas H16, Hydrogenomonas eutropha) was described to be activated by FMN about 2-3 fold (20-22) or even up to 15 fold (23). Present results thus indicate (i) that a certain portion of FMN,

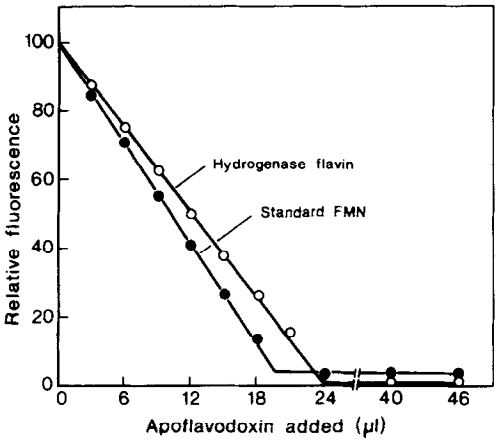

Fig. 2. Fluorimetric titration of FMN with apoflavodoxin. Increments of apoflavodoxin (20.7 µM) were added to solutions of 1 µM standard FMN and unknown concentration of hydrogenase flavin, respectively and the fluorescence was measured after each addition (excitation 445 nm, emission 525 nm). ● — ● standard FMN; o —— o flavin of hydrogenase.

TABLE 2

FMN CONTENT OF DIFFERENT PREPARATIONS OF PURIFIED HYDROGENASE CORRELATED TO THE ACTIVITY INCREASE EXERTED BY THE ADDITION OF FMN

Enzyme preparation	Specific activity (µmol NAD reduced/min · mg protein)			FMN content (mol/mol of enzyme)	
	without addition	addition of FMN (1 µM)	% activation by added FMN	as determined	as expected from the percentage of activation by FMN
1	36.4	65.5	80	1.12	2.02
2	45.0	71.0	58	1.20	1.90
3	47.5	71.3	50	1.37	2.06

which is needed for maximal hydrogenase activity, dissociates from the enzyme and gets lost during preparation and (ii) that the values determined for FMN content of hydrogenase are too low.

Assuming that the percentage of activation by added FMN corresponds to the amount of FMN removed from hydrogenase during purification, a corrected FMN content was calculated yielding values close to 2.0 mol/mol of enzyme in all preparations. In conclusion, the data summarized in Table 2, exhibiting obvious correlations between specific activity, percentage of enzyme activation by added FMN and FMN content of enzyme, strongly suggests that hydrogenase indeed contains 2 FMN per molecule. From iron and acid-labile sulfide analysis (20) we can conclude that the ratio FMN : iron : sulfide in hydrogenase is 2 : 12 : 12. For additional confirmation of these statements investigations on deflavoenzyme and reconstitution of native hydrogenase including determination of FMN in preparations of the fully reconstituted enzyme are recommendable. However, preliminary studies revealed that complete enzyme reactivation and incorporation of FMN into the protein only occurs using the reduced enzyme. As hydrogenase when exposed to reducing conditions becomes unstable and denaturates easily (12), preparations of reconstituted hydrogenase appear to be not suitable for the use of exact quantitative determinations.

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REFERENCES

1. Chen, J.S. and Mortenson, L.E. (1974) Biochim. Biophys. Acta 371, 283-298.
2. Erbes, D.L., Burris, R.H., and Orme-Johnson, W.H. (1975) Proc.Nat.Acad.Sci. USA 72, 4795-4799.
3. Chen, J.S., Mortenson, L.E., and Palmer, G. (1976) Iron and Copper Proteins (Yasonobo, K.T., and Mower, H.F., eds.), pp. 68-81, Plenum Press, New York.
4. LeGall, J., Dervartanian, D.V., Spilker, E., Lee, J.-P., and Peck, Jr., H.D. (1971) Biochim. Biophys. Acta 234, 525-530.
5. Haschke, R.H. and Campbell, L.L. (1971) J. Bacteriol. 105, 249-258.

6. Yagi, T., Kimura, K., Daidoji, H., Sakai, F., Tamura, S., and Inokuchi, H. (1976) J. Biochem. 79, 661-671.
7. van der Westen, H.M., Mayhew, S.G., and Veeger, C. (1978) FEBS Letters 86, 122-126.
8. Gitlitz, P.H. and Krasna, A.J. (1975) Biochemistry 14, 2561-2568.
9. Kakuno, T., Kaplan, N.O., and Kamen, M.D. (1977) Proc. Nat. Akad. Sci. USA 74, 861-863.
10. Gogotov, I.N., Zorin, N.A., Serebriakova, L.T., and Kondratieva, E.N. (1978) Biochim. Biophys. Acta 523, 335-343.
11. Adams, M.W.W., and Hall, D.O. (1977) Biochem. Biophys. Res. Commun. 77, 730-737.
12. Schneider, K. and Schlegel, H.G. (1976) Biochim. Biophys. Acta 452, 66-80.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Beisenherz, G., Bolze, H.J., Bücher, T., Czok, R., Garbade, K.H., Meyer-Arendt, E., and Pfleiderer, G. (1953) Z. Naturf. 8B 555-577.
15. Mayhew, S.G., and Massey, V. (1969) J. Biol. Chem. 244, 794-802.
16. Wassink, J.H., and Mayhew, S.G. (1975) Anal. Biochem. 68, 609-616.
17. Mayhew, S.G. (1971) Biochim. Biophys. Acta 235, 289-302.
18. Bessey, O.A., Lowry, O.H., and Love, R.H. (1949) J. Biol. Chem. 180, 755-769.
19. Mayhew, S.G., and Strating, M.J.J. (1975) Eur.J. Biochem. 59, 539-544.
20. Repaske, R., and Seward, C. (1960) Biochem. Biophys. Res. Commun. 2, 397-401.
21. Eberhardt, U. (1966) Arch. Mikrobiol. 53, 288-302.
22. Repaske, R., and Dans, C.L. (1968) Biochem. Biophys. Res. Commun. 30, 136-141.
23. Repaske, R. (1962) J. Biol. Chem. 237, 1351-1355.
24. Schneider, K., and Cammack, R. (1978) Hydrogenases: Their Catalytic Activity, Structure and Function (Schlegel, H.G., ed.) E. Goltze KG, Göttingen, in press.