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CHARACTERIZATION AND SPECTROSCOPIC PROPERTIES OF REDUCED Mo AND W FORMATE DEHYDROGENASE FROM C. THERMOACETICUM

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SUMMARY: Formate dehydrogenase (FDH) (EC 1.2.1.43) from C. thermoaceticum has been purified in two forms. One contains tungsten (W), and the other is enriched in molybdenum (Mo). The W-FDH is clearly active, while the Mo results are ambiguous with enzymatic activities generally lower in the Mo-enriched samples. Spectroscopic studies (EPR, absorption, and CD) on W-FDH and Mo-FDH demonstrate that no signal correlates to the group VI metal active site in the dithionite-reduced enzyme. This lack of a W(V) EPR signal is in contrast to the results observed for tungsten-substituted sulfite oxidase which is inactive.

While a number of extremely important metalloenzymes contain molybdenum (Mo) at their active sites, replacement with tungsten (W) is known to abolish activity of sulfite oxidase [1], xanthine oxidase [2], nitrate reductase [3], and nitrogenase [4]. The effect of tungsten on formate dehydrogenase (FDH) is more varied. The nitrate reductase-coupled FDH of E. coli is similar to the Mo-proteins above in that tungsten incorporation eliminates catalytic activity [5]. M. vannelli produces two different FDH's depending on whether or not the growth culture is supplemented with tungsten (and selenium) [6]. Exclusion of tungsten results in an Mo-FDH of approximately 105,000 daltons, while supplementation with tungsten and selenium results in an FDH with substantially higher molecular weight. However, the catalytic role of tungsten in this protein has yet to be defined. In Clostridia, the FDH endogenous to C. pasteurianum has been shown to be exclusively an Mo-protein with no change in catalytic activity upon tungsten addition [7], while nutritional studies at the level of crude cell

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extract have indicated that addition of tungsten to the growth media depresses FDH activity in C. purinolyticum [8] and C. cylindrosporum [9]. In contrast, tungsten addition enhances FDH activity in C. acidiurici [9], C. formiaceticum [10], and C. thermoaceticum [11]. The extreme oxygen sensitivity of these FDH's has impeded their purification to homogeneity and thus precluded direct evaluation of the group VI metal requirement for catalysis and detailed study of the group VI metal active site.

Following the recent purification procedure of Ljungdahl which yields a highly purified soluble FDH from C. thermoaceticum [12], we have prepared tungsten- and molybdenum-enriched forms of the enzyme. We report here preliminary physico-chemical characterization of the dithionite-reduced form of these enzymes.

MATERIALS AND METHODS

C. thermoaceticum (ATCC 39073) was grown and harvested as previously reported [13], except the single group VI metal culture media contained either 21 μM sodium tungstate without molybdenum or 42 μM ammonium paramolybdate with no tungsten. The native cells (grown on media containing 10 μM molybdenum and 10 μM tungsten) were a gift of Prof. L. Ljungdahl. The FDH isoenzymes were isolated and purified under a 95% N_2 and 5% H_2 atmosphere as described by Ljungdahl [12]. The enzymatic reaction rate was determined anaerobically by monitoring the production of NADPH spectrophotometrically in the presence of 40 mM formate and 1 mM $NADP^+$ at 50° as described previously [13]. Protein concentrations were determined by the method of Bradford [14] employing bovine serum albumin as the standard. FDH concentrations were determined by dividing the aqueous protein concentration by the FDH molecular weight of 340,000 g/mole [13]. Thus, specific activity units are expressed as μmoles of NADPH formed per minute per milligram of protein. Analytical electrophoresis was performed by the method of Brewer and Ashworth [15] modified for anaerobic proteins by Ljungdahl [12]. With the exception of ultra-high purity GTE Sylvania (Towanda, Pa) sodium tungstate and ammonium paramolybdate, all other reagents were purchased in the highest commercial purity available. Tungsten analyses of the purified enzymes were performed on an Applied Research Laboratories 35000 C Inductively Coupled Plasma Spectrophotometer. Iron and molybdenum contents were determined with a graphite furnace atomic absorption spectrometer, Perkin-Elmer model 2380 and HGA-400 furnace controller. Optical absorption spectra were taken on a Cary 17 spectrophotometer, and CD spectra on a Jasco J-500 C spectropolarimeter at room temperature. EPR spectra were recorded with a Brüker ER-220D-SRC EPR spectrometer at 77K and at 10K using an Air Products LTD-3-110 Heli-Tran liquid helium transfer refrigerator. Sample transfers were done in a Vacuum Atmospheres Dribox. All spectral studies reported were performed in the presence of 2 mM dithionite.

RESULTS AND DISCUSSION

Table 1 presents the elemental composition of six formate dehydrogenase (FDH) samples which are representative of 12 preparations. Group VI metal

Table 1: Elemental analyses and specific activities for six formate dehydrogenase preparations.

Preparation	Metal Analyses (mole per mole FDH) Mo	W	Fe	Specific Activity (SPAC) μmole NADPH min^{-1} mg^{-1}	SPAC / Group VI	SPAC / W
W-3	.08	1.76	48.1	1092	593	620
W-5	.05	.71	9.5	670	882	943
Mo-2	1.69	<.31	41.5	331	195	--
Mo-3	.49	1.0	45.6	593	398	593
Native-1	.19	.92	32.0	548	494	596
Native-2	.20	.34	26.7	334	619	982

analyses of the enzymes isolated from tungsten growth media indicate that the W-FDH derivative contains less than 0.08 mole of molybdenum per mole of enzyme. The catalytic activity of these proteins increases with tungsten content. The highest activity enzyme sample isolated possessed a specific activity of 1,092 units and 1.76 moles of tungsten per mole of enzyme. Both of these proteins were homogeneous by the criterion of single electrophoretic bands after protein and FDH activity staining.

Group VI metal analyses of the FDH's isolated from molybdenum-supplemented bacteria show an enhanced molybdenum content in comparison with the tungsten-enriched and molybdo-tungsten (i.e., native) proteins. However, in contrast to the results of the Mo-free, W-only FDH preparations we did not obtain a W-free, Mo-only FDH, in spite of the cells being maintained on the normal growth media with 42 μM molybdate and no tungsten for over one year prior to protein purification. The presence of tungsten in these samples demonstrates the high affinity of this Clostridial FDH for tungsten and the inherent difficulty in isolating a pure Mo-FDH. Group VI analyses of the molybdo-tungsten (i.e., native) enzyme illustrate that both metals are present in native FDH. Although a quantitative correlation between specific activity and molybdenum mole

fraction is unclear, activities are generally lower in the molybdenum-enriched proteins.

Comparison of the iron content of these proteins reveals a considerable variance, even in those proteins which were judged homogeneous by analytical electrophoresis (W-5, W-3, and Mo-3). Iron content variability in different enzymatic samples was also observed by Ljungdahl [12] and during the purification of nitrate reductase [16]. In the latter case, the extra iron was believed to be adventitiously bound so as to elute with the protein. Clearly, the variability in iron content between different preparations does not allow identification of the number of catalytically relevant iron atoms. In addition, the presence of non-catalytic iron requires an additional caveat in interpreting spectra relating to iron chromophores.

The elemental composition of the tungsten-enriched FDH's supports the results of Ljungdahl that a fully active FDH should contain two tungsten atoms. The activity per group VI metal atom, also listed in Table 1, suggests a maximum enzymatic activity between 1,200-1,800 specific activity units which is in good agreement with the published literature [12]. The role of molybdenum and its activity relative to tungsten in this protein remains uncertain. However, it does appear that Mo-enriched FDH's exhibit consistently lower activities than the tungsten form, and that small amounts of tungsten compete effectively for active site incorporation even in the presence of a large excess of molybdenum.

In its dithionite-reduced form, FDH is EPR silent. Spectra were recorded at 77K and 10K over a range of microwave powers from 1 to 200 mW. The most active samples exhibited no EPR signal. However, less pure and less active samples exhibited small signals at high instrument gain with g values 2.03-2.05, 2.01, 1.94, and 1.89. Figure 1 shows the g=2 region of a fraction from the W-3 preparation which eluted immediately following the main fraction, and is representative of EPR spectra sometimes seen in FDH preparations. However, the main W-3 fraction, which was pure by electrophoresis, had no EPR signal. The highest specific activity fractions of W-5, Mo-2, Mo-3, and Native-2 preparations were

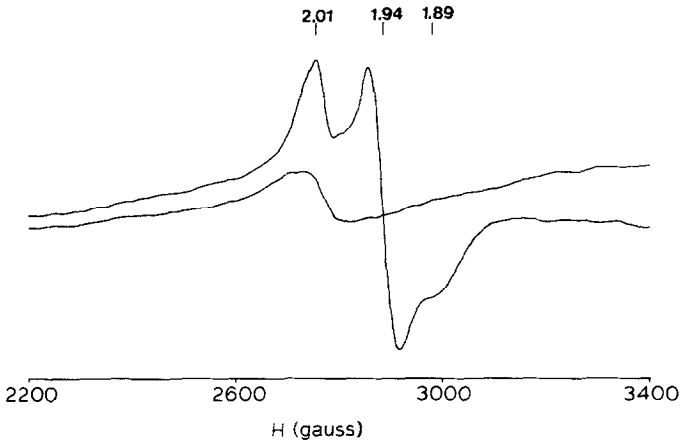

Figure 1: EPR spectrum of a W-3 preparation fraction which immediately followed the main fraction. Conditions: 10K, 124 mW, 9.45 GHz, gain 2.5 x 10^4. Note dewar impurity signal in the baseline spectrum.

also all EPR silent. Thus, the EPR signals that are occasionally observed derive from either small amounts of contaminating iron-sulfur proteins or partly oxidized FDH.

Figure 2 contains the absorption and CD spectra of the 300-850 nm region for several Mo- and W-FDH preparations. The absorption spectrum from 300-1,200 nm is broad and featureless with very high ε values (1.6 x 10^5 $M^{-1}cm^{-1}$ at 390 nm). This is consistent with a large number of iron-sulfur centers. While the same general features are found in the CD spectra of all FDH samples, there are significant differences in the relative intensities between enzymatic preparations. These differences do not correlate with group VI metal content or activity, but are apparently due to some inhomogeneity even at the level of purity defined by electrophoresis above. No correlation between the variation in CD intensity and the spurious EPR signals could be recognized. The CD features are dominantly due to iron-sulfur clusters, but CD spectra are not diagnostic of specific cluster type.

While it is not surprising that absorption and CD spectra do not correlate with the group VI metal due to the dominance of the iron-sulfur centers, the lack of a W(V) EPR signal is significant. Reduction of tungsten-substituted sulfite oxidase has been reported to produce a W(V) EPR detectable state which

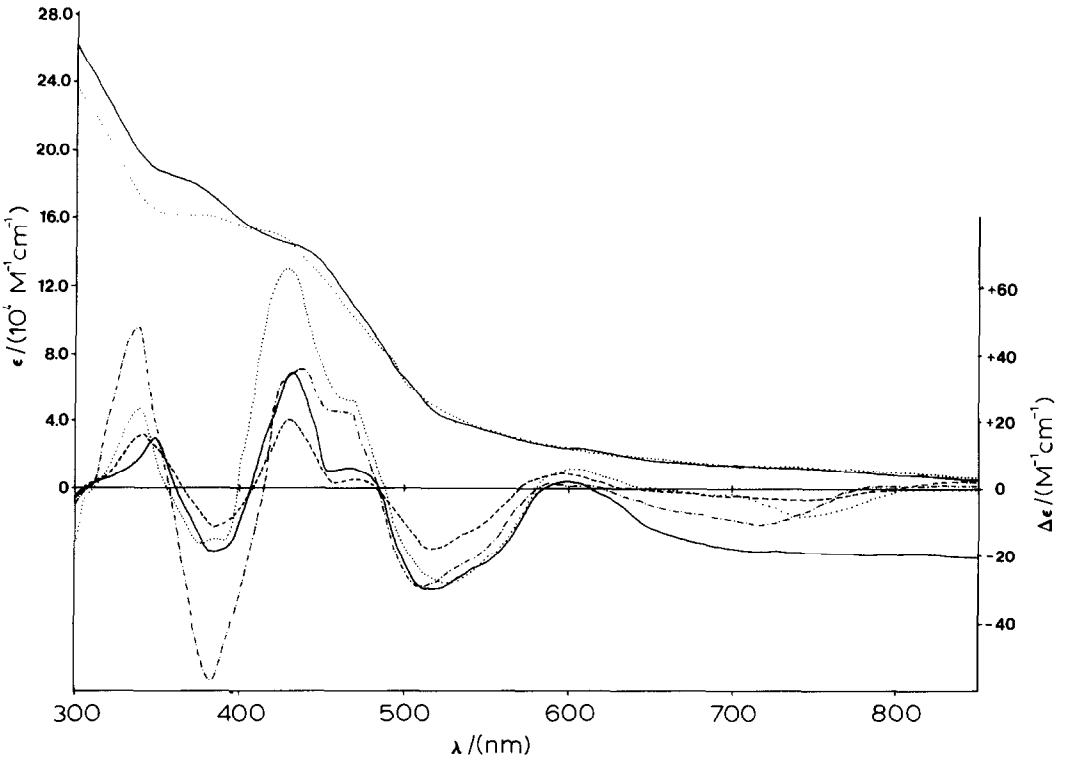

Figure 2: Absorption and CD spectra of several Mo- and W-FDH preparations. Absorption: W-3, —— ; Mo-2, ···· . CD: W-3, —— ; Mo-2, ···· ; W-5, ---- ; Mo-3, -·-·- .

is not capable of further reduction [17]. This apparently relates to the inability of the tungsten-substituted sulfite oxidase to undergo catalytic turnover since tungsten cannot cycle between the VI and IV oxidation states as the molybdenum enzyme does. Thus, the lack of an EPR signal from W-FDH in its dithionite-reduced form indicates that if an analogous active site cofactor exists in this enzyme, either tungsten remains in the VI oxidation state even during turnover, or more likely, that it is capable of being reduced below the V state. Elucidation of the ligands and electronic structure of the tungsten active site in FDH should provide insight into how this tungsten active site complex functions in redox catalysis.

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REFERENCES

1. Johnson, J. L., Cohen, H. J., and Rajagopalan, K. V. (1974) J. Biol. Chem. 249, 5046.
2. Johnson, J. L., Waud, W. R., Cohen, H. J., and Rajagopalan, K. V. (1974) J. Biol. Chem. 249, 5056.
3. Enoch, H. G., and Lester, R. L. (1972) J. Bacteriol. 110, 1032.
4. Nagatani, H. H., and Brill, W. J. (1974) Biochim. Biophys. Acta 362, 160.
5. Scott, R. H., and Demoss, J. A. (1976) J. Bacteriol. 126, 478.
6. Jones, J. B., and Stadtman, T. C. (1981) J. Biol. Chem. 256, 656.
7. Scherer, P. A., and Thauer, R. K. (1978) Eur. J. Biochem. 85, 125.
8. Durre, P., Andersch, W., and Andreesen, J. R. (1981) International Journal of Systematic Bacteriology 31, 184.
9. Wagner, R., and Andreesen, J. R. (1977) Arch. Microbiol. 114, 219.
10. Leonhardt, U., and Andreesen, J. R. (1977) Arch. Microbiol. 115, 277.
11. Andreesen, J. R., and Ljungdahl, L. G. (1973) J. Bacteriol. 116, 867.
12. Yamamoto, I., Saiki, T., Liu, S. M., and Ljungdahl, L. G. (1983) J. Biol. Chem. 258, 1826.
13. Ljungdahl, L. G., and Andreesen, J. R. (1978) Methods in Enzymology 53, 360.
14. Bradford, M. M. (1976) Anal. Biochem. 72, 248.
15. Brewer, J. M., and Ashworth, R. B. (1969) J. Chem. Ed. 46, 41.
16. Jacob, G. S., and Orme-Johnson, W. H. (1980) in "Molybdenum and Molybdenum-Containing Enzymes," p. 331 (Ed. M. Coughlin).
17. Johnson, J. L., and Rajagopalan, K. V. (1976) J. Biol. Chem. 251, 5505.