Biochimica et Biophysica Acta, 1100 (1992) 105–108

BBABIO 40260

Rapid Report

Oxidation-reduction properties of the ferredoxin-linked glutamate synthase from spinach leaf

Masakazu Hirasawa [a], Dan E. Robertson [b], Emmanuel Ameyibor [c], Michael K. Johnson [c] and David B. Knaff [a]

[a] *Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX,* [b] *Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, PA and* [c] *Department of Chemistry, University of Georgia, Athens, GA (USA)*

(Received 28 January 1992)

Key words: Glumate synthase; Ferredoxin; Oxidation; Reduction

Oxidation-reduction titrations have been conducted to determine the midpoint potential (E_m) values of the three electron-carrying prosthetic groups of the ferredoxin-linked glutamate synthase isolated from spinach leaves. Titrations using electron paramagnetic resonance (EPR) signals to monitor the oxidation state of the [3Fe-4S]$^{+,0}$ cluster found in the enzyme, indicated the presence of a single $n = 1$ component with $E_m = -170$ mV at pH 7.7. Titrations using absorbance changes in the visible region to monitor the oxidation states of the FAD and FMN groups present in the enzyme could be fit to a single $n = 2$ Nernst curve with $E_m = -180$ mV at pH 7.7. The magnitude of the absorbance change observed during this titration accounts for all of the FMN and FAD found in the enzyme, indicating that the two flavins are either isopotential or differ in E_m by less than about 30 mV. Neither optical nor EPR titrations gave any evidence for the presence of stable flavin free radicals. These results represent the first characterization of the redox properties of the prosthetic groups of a ferredoxin-dependent glutamate synthase.

Introduction

The assimilation of inorganic nitrogen by photosynthetic eukaryotes involves the sequential reduction of nitrate to nitrite and then of nitrite to ammonia, catalyzed by the enzymes nitrate reductase and nitrite reductase, respectively, followed by the incorporation of ammonia into glutamate to form glutamine and the subsequent reductive conversion of glutamine plus 2-oxoglutarate to form two molecules of glutamate [1,2]. The last step in this portion of the pathway, resulting in the formation of glutamate, is catalyzed by the soluble enzyme glutamate synthase (EC 1.4.7.1). The glutamate synthase-catalyzed reaction occurs in the chloroplast stroma and uses reduced ferredoxin to provide the two electrons required for the reductive conversion of 2-oxoglutarate plus glutamine to glutamate [2]. Glutamate synthases isolated from a large number of prokaryotes and eukaryotes use reduced pyridine nucleotides as the electron donor [3], as do several glutamate synthases found in non-green plant tissues [2,4–8], but chloroplast-located glutamate synthases are unique in requiring reduced ferredoxin as the physiological electron donor. Spinach leaf glutamate synthase forms an electrostatically stabilized complex with ferredoxin that involves negatively charged groups on ferredoxin and that appears to involve binding domains similar to those present in complexes of ferredoxin with other chloroplast enzymes [2,9–11].

Monomeric ferredoxin-dependent glutamate synthases, with molecular masses near 160 kDa, have been purified to homogeneity from spinach leaves [12] and from the green alga *Chlamydomonas reinhardtii* [13,14] and in each case shown to contain one FMN and one FAD per enzyme molecule. Non-heme iron and acid-labile sulfide analyses [12,14] indicated the likely presence of an iron-sulfur cluster in the spinach leaf and *C. reinhardtii* enzymes and recently evidence has been obtained from EPR, magnetic circular dichroism (MCD) and Resonance Raman spectroscopies establishing the presence of a single [3Fe-4S]$^{+,0}$ cluster in the spinach enzyme [15]. An EPR spectrum characteristic of a [3Fe-4S]$^{+}$ cluster has also been observed in

Abbreviations: E_m, oxidation-reduction midpoint potential vs. the normal hydrogen electrode; EPR, electron paramagnetic resonance; HiPIP, high potential iron protein; MCD, magnetic circular dichroism; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

Correspondence: D.B. Knaff, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, TX 79409-1061, USA.

samples of purified NAD(P)H-linked glutamate synthases isolated from *Escherichia coli* [16] and from the diazotrophs *Azospirillum brasilense* [17] and *Azotobacter vinelandii* [16]. Amino acid sequences have been deduced from the corresponding gene sequences for the monomeric (M_r = 165 298), ferredoxin-dependent maize glutamate synthase [18] and for the large subunit of the NAD(P)H-dependent *E. coli* enzyme [19] and both sequences show patterns of cysteine spacings consistent with the presence of a function $[3Fe\text{-}4S]^{+,0}$ cluster (see Ref. 15 for a discussion) and regions likely to be involved in flavin binding [18,19]. Despite the availability of this considerable knowledge about glutamate synthases, little is known about the thermodynamic oxidation-reduction properties of the prosthetic groups of this important class of enzymes. We present below the first determinations of E_m values for all three prosthetic groups of the ferredoxin-dependent spinach enzyme.

Spinach glutamate synthase and ferredoxin were purified from commercially obtained spinach leaves and their concentrations estimated as previously described [10]. Protein concentration was estimated according to the method of Bradford [20] using bovine serum albumin as a standard. The optical absorbance and EPR spectra and turnover numbers, with either reduced methyl viologen or reduced ferredoxin as electron donor to the glutamate synthase preparation were essentially identical to those reported previously [9–12,15]. The glutamate synthase sample used for these experiments showed a single Coomassie brilliant blue-staining band, corresponding to 160 kDa, after polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) using a Pharmacia Phast Electrophoresis System. Oxidation-reduction titrations using an optically transparent gold mesh electrode were carried out as described previously [21], using an Aminco DW-2a spectrophotometer to monitor absorbance changes. Oxidation-reduction titrations of flavin absorbance changes using potassium ferricyanide and sodium dithionite as titrants were conducted according to the method of Dutton [22], as described previously [23]. Oxidation-reduction titrations of the $[3Fe\text{-}4S]^{+,0}$ cluster were performed at ambient temperature under argon in a Vacuum Atmospherics glove box, with samples first fully reduced with a slight excess of sodium dithionite and then oxidized by titration with potassium ferricyanide. After equilibration at the desired potential, a 0.2 ml aliquot was transferred to a calibrated EPR tube and immediately frozen in liquid nitrogen. Potentials, which were measured with a platinum working electrode and a saturated calomel reference electrode, are reported vs. the normal hydrogen electrode. X-band EPR spectra of the $[3Fe\text{-}4S]^{+}$ state of the cluster were obtained and recorded as described previously [15]. Oxidation-reduction titrations on samples containing no glutamate synthase, but only buffer and redox mediators, were conducted for both the optical and EPR experiments to insure that none of the signals attributed to the enzyme prosthetic groups originated instead from the mediators.

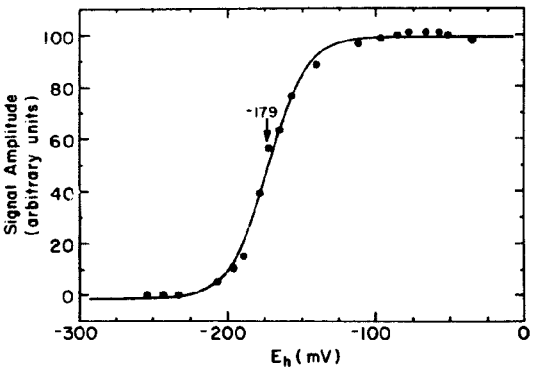

Fig. 1. Oxidation-reduction titration of the FMN and FAD groups of spinach glutamate synthase. The reaction mixture contained 5.0 μM glutamate synthase in 100 mM Tricine buffer (pH 7.7) containing 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% β-mercaptoethanol and the following oxidation-reduction mediators: 2 μM 2-hydroxy-1,4-naphthoquinone; 2 μM anthraquinone-2-sulfonate; 10 μM pyocyanine and 2 μM benzyl viologen. The extent of flavin reduction was monitored by following the absorbance at 444 nm minus 522 nm. The data were fitted to the Nernst equation for a single two-electron electron carrier with $E_m = -179$ mV.

Initial attempts to perform oxidation-reduction titrations on the FMN and FAD groups of glutamate synthase using an optically transparent gold electrode proved unsuccessful due to substantial protein denaturation at the electrode surface. Thus, titrations were conducted using sodium dithionite as a chemical reductant and potassium ferricyanide as a chemical oxidant. The results of one such titration are shown in Fig. 1. The data from several titrations gave good fits to the Nernst Equation for a single $n = 2$ component with $E_m = -180 \pm 10$ mV at pH 7.7. These titrations were conducted in the presence of 200 mM NaCl, 1 mM 2-oxoglutarate and 0.1% (v/v) β-mercaptoethanol, conditions known to increase the stability of the enzyme [11,12]. No absorbance changes suggestive of the presence of either anionic or neutral flavin semiquinones were detected during the course of the titrations. Attempts to fit the titration data to two $n = 2$ components, so that the E_m values of the FMN and FAD prosthetic groups might be distinguished, did not improve the fit. Oxidized minus reduced difference spectra (see Fig. 2 for an example) taken at several different points during the titrations were essentially identical, as would be expected if the two flavins were isopotential or differed in E_m values by < 30 mV. Of course, similar difference spectra would also be obtained for different portions of the titration if only one

of the two flavins present in the enzyme were being titrated. However, the total magnitude of the absorbance change recorded during the titrations (i.e., the difference in absorbance between samples poised at $E_h > +100$ mV and at $E_h < 250$ mV) was identical to that observed when the fully oxidized enzyme was completely reduced by an excess of sodium dithionite, indicating that the titrations correspond to the complete reduction of both FMN and FAD groups present in the enzyme. Thus, it appears that the FMN and FAD groups of spinach glutamate synthase differ by no more than 30 mV in E_m values.

Oxidation-reduction titrations of the $[3Fe\text{-}4S]^{+,0}$ were conducted using the EPR signal of the oxidized cluster [15] to monitor its oxidation state. The results of one such titration are shown in Fig. 3. Titrations gave good fits to the Nernst equation for a single $n = 1$ component with $E_m = -170 \pm 15$ mV at pH 7.7. As in the case of the flavin optical titrations described above, the titrations of the $[3Fe\text{-}4S]^{+,0}$ cluster were conducted at high ionic strength, in the presence of β-mercaptoethanol and 2-oxoglutarate, to increase the stability of the enzyme. No evidence for any additional iron-sulfur clusters was observed in the EPR spectra obtained during the course of the titration and the magnitude of the EPR signal observed in fully oxidized samples (i.e., at $E_h > -75$ mV) corresponded to one spin per enzyme molecule. No EPR signals that could be attributed to the presence of a flavin free radical were observed in any of the titration samples.

The results presented above comprise the first information available on the E_m values of the prosthetic groups in a ferredoxin-linked glutamate synthase and provide an essential thermodynamic base for future mechanistic studies. It is noteworthy that all three of the electron-carrying groups in the enzyme are approximately isopotential. As it appears that the mechanism by which the enzyme functions requires a block of isopotential carriers, it is likely that the choice of the $[3Fe\text{-}4S]^{+,0}$ form of an iron-sulfur cluster as an indigenous electron carrier in spinach glutamate synthase is due to redox potential considerations. $[3Fe\text{-}4S]^{+,0}$ centers generally exhibit E_m values intermediate between those of the [2Fe-2S] and $[4Fe\text{-}4S]^{2+,1+}$ ferredoxin-type clusters, which have potentials ranging from −300 to −600 mV, and those of the HiPIP-type $[4Fe\text{-}4S]^{3+,2+}$ clusters, which have E_m values near +350 mV [24].

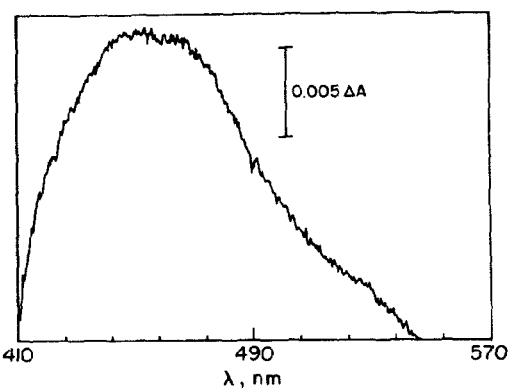

Fig. 2. Oxidized minus reduced difference spectrum for glutamate synthase flavins. The difference spectrum, obtained from data collected during the titration shown in Fig. 1, was generated by subtracting the spectrum of the enzyme at $E_h = -233$ mV from the spectrum at $E_h = -189$ mV.

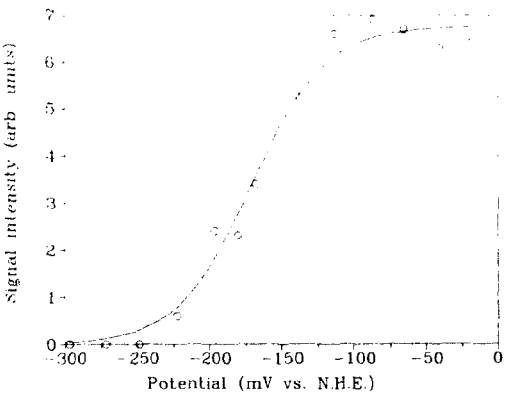

Fig. 3. EPR titration of the [3Fe-4S] center in spinach glutamate synthase. The samples contained 62 μM enzyme in the same buffer used for the titration of Fig. 1, except that the following mediators were used, all at a concentration of 50 μM: neutral red; safranin; phenosafranin; anthraquinone-1,5-disulfonate; 2-hydroxy-1,4-naphthoquinone; indigo-disulfonate; methylene blue and 1,4-naphthoquinone. EPR spectra were recorded at 10 K with a microwave power of 10 mW, modulation amplitude of 0.63 mT and a microwave frequency of 9.43 GHz. The signal intensity represents the peak-to-trough magnitude ($g = 2.02$ to $g = 1.94$) of the oxidized $[3Fe\text{-}4S]^{+}$ center (in arbitrary units) measured at different potentials. The solid line represents the best fit to the Nernst equation for a one-electron carrier with $E_m = -170$ mV.

This work was supported by grants from the National Institutes of Health (GM33806 to M.K.J. and GM27309 to P.L. Dutton), the United States Department of Energy (DE-FG05-90ER20017 to D.B.K.) and the Robert A. Welch Foundation (D-0710 to D.B.K.).

References

1 Guerrero, M.G., Vega, J.M. and Losada, M. (1981) Annu. Rev. Plant Physiol. 32, 169–204.
2 Knaff, D.B. and Hirasawa, M. (1991) Biochim. Biophys. Acta 1056, 93–125.
3 Vanoni, M.A., Curti, B. and Zanetti, G. (1991) in Chemistry and Biochemistry of Flavoenzymes, Vol. 3 (Muller, F., ed.), pp. 309–317, CRC Press, Boca Raton.
4 Suzuki, A., Vidal, J. and Gadal, P. (1982) Plant Physiol. 70, 827–831.
5 Suzuki, A. and Gadal, P. (1984) Physiol. Veg. 22, 471–486.
6 Matoh, T., Ida, S. and Takahashi, E. (1980) Plant Cell Physiol. 21, 1461–1474.

7 Chen, E.L. and Cullimore, J.V. (1988) Plant Physiol. 88, 1411–1417.
8 Anderson, M.P., Vance, C.P., Heichel, G.H. and Miller, S.S. (1989) Plant Physiol. 90, 351–358.
9 Hirasawa, M., Boyer, J.M., Gray, K.A., Davis, D.J. and Knaff, D.B. (1986) Biochim. Biophys. Acta 851, 23–28.
10 Hirasawa, M., Chang, K.-T., Morrow, K.J., Jr. and Knaff, D.B. (1989) Biochim. Biophys. Acta 977, 150–156.
11 Hirasawa, M., Chang, K.-T. and Knaff, D.B. (1991) Arch. Biochem. Biophys. 286, 171–177.
12 Hirasawa, M. and Tamura, G. (1984) J. Biochem. 95, 983–994.
13 Galván, F., Márquez, A.J. and Vega, J.M. (1984) Planta 162, 180–187.
14 Márquez, A.J., Gotor, C., Romero, L.C., Gálvan, F. and Vega, J.M. (1986) Int. J. Biochem. 18, 531–535.
15 Knaff, D.B., Hirasawa, M., Ameyibor, E., Fu, W. and Johnson, M.K. (1991) J. Biol. Chem. 266, 15080–15084.
16 Rendina, A. (1980) Ph. D. Thesis, University of Wisconsin.
17 Vanoni, M.A., Zanetti, G., Curti, B. and Edmondson, D.E. (1991) in Flavins and Flavoproteins 1990 (Curti, B., Ronchi, S. and Zanetti, G., eds.), pp. 749–753, Walter de Gruyter, Berlin.
18 Sakakibara, H., Watanabe, M., Hase, T. and Sugiyama, T. (1991) J. Biol. Chem. 266, 2028–2035.
19 Oliver, G., Gosset, G., Sanchez-Pescador, R., Lozoya, E., Ku, L.M., Flores, N., Becerril, B., Valle, F. and Bolivar, F. (1987) Gene 60, 1–11.
20 Bradford, M.M. (1976) Anal. Biochem. 72, 248–259.
21 Smith, J.M., Smith, W.H. and Knaff, D.B. (1981) Biochim. Biophys. Acta 635, 405–411.
22 Dutton, P.L. (1978) Methods Enzymol. 54, 411–435.
23 Güner, S., Robertson, D.E., Yu, L., Qiu, Z., Yu, C.-A. and Knaff, D.B. (1991) Biochim. Biophys. Acta 1058, 269–279.
24 Ackrell, B.A., Johnson, M.K., Gunsalus, R.P. and Cecchini, G. (1991) in Chemistry and Biochemistry of Flavoenzymes, Vol. III (Muller, F., ed.), pp. 229–297, CRC Press, Boca Raton.