A Loop Unique to Ferredoxin-Dependent Glutamate Synthases is Not Essential for
Ferredoxin-Dependent Catalytic Activity

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Abstract

  It had been proposed that a loop, typically containing 27 amino acids, which is only present in monomeric, ferredoxin-dependent, "plant-type" glutamate synthases and is absent from the catalytic α–subunits of both NADPH-dependent, heterodimeric glutamate synthases found in non-photosynthetic bacteria and NADH-dependent heterodimeric cyanobacterial glutamate synthases, plays a key role in productive binding of ferredoxin to the plant-type enzymes. Site-directed mutagenesis has been used to delete the entire 27 amino acid-long loop in the ferredoxin-dependent glutamate synthase from the cyanobacterium *Synechocystis* sp. PCC 6803. The specific activity of the resulting loopless variant of this glutamate synthase, when reduced ferredoxin serves as the electron donor, is actually significantly higher than that of the wild-type enzyme, suggesting that this loop is not essential for efficient electron transfer from reduced ferredoxin to the enzyme. Furthermore, the binding affinity of the loopless variant for formation of a complex between the enzyme and ferredoxin is more than 2-fold greater than that of the wild-type enzyme. These results are consistent with the results of an *in silico* modeling study that suggests that the loop is unlikely to comprise a part of the ferredoxin-binding domain of the enzyme.

Keywords: glutamate synthase; ferredoxin; protein/protein interactions; FMN; iron-sulfur clusters

Abbreviations: CD, circular dichroism; Fd, ferredoxin; FMN, flavin mononucleotide; GOGAT, glutamine:oxoglutarate amidotransferase; ITC, isothermal titration calorimetry; MALDI-TOF, matrix-assisted laser desorption ionization, time-of-flight; MS, mass spectrometry; MV, methyl viologen; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate

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Introduction

      The enzyme glutamine:oxoglutarate amidotransferase (often referred to simply as glutamate synthase and hereafter abbreviated as GOGAT), catalyzes the 2-electron reductive conversion of one molecule of 2-oxoglutarate plus one molecule of glutamine to form two molecules of glutamate, and plays a key role in nitrogen assimilation in oxygenic photosynthetic organisms (Hase et al. 2006; Suzuki and Knaff 2005; Vanoni and Curti, 1999; Vanoni and Curti 2008). The "plant-type" GOGATs (EC 1.4.7.1), present in cyanobacteria and in the stromal space of chloroplasts in algae and flowering plants, are soluble, monomeric proteins with molecular masses of approximately 170 kDa (Hase et al. 2006; Suzuki and Knaff 2005; Vanoni and Curti, 1999; Vanoni and Curti 2008). They contain a single [3Fe-4S] cluster and a single FMN as the sole prosthetic groups (Hase et al. 2006; Suzuki and Knaff 2005; Vanoni and Curti, 1999; Vanoni and Curti 2008). Although reduced methyl viologen (hereafter abbreviated as MV) can serve as an effective non-physiological electron donor in the reaction catalyzed by these enzymes, they are specific for reduced ferredoxin (hereafter abbreviated as Fd) as the physiological electron donor and display no activity with either NADH or NADPH as an electron donor (Hase et al. 2006; Navarro et al. 2000; Suzuki and Knaff 2005; Vanoni and Curti 2008). Not surprisingly, given the need for GOGAT to interact productively with its electron-donating substrate, substantial evidence has been obtained documenting that several Fd-dependent GOGATs, including enzymes from flowering plants, algae and cyanobacteria, can form a complex (K_d = ca. 1 to 50 µM) with Fd at low ionic strength (García-Sanchez et al. 1997; García-Sanchez et al. 2000; Schmitz et al.1996; Hirasawa

and Knaff 1986; Hirasawa et al. 1986; Hirasawa et al. 1989; Hirasawa et al. 1991; Sbinmura et al. 2012; van den Heuvel et al. 2003). The observation that the complexes form at low ionic strength and dissociate at higher ionic strength has been interpreted as indicating a significant contribution of electrostatic forces in stabilizing the complex (Hirasawa et al. 1986; Hase et al. 2006).

In contrast, GOGATs from non-photosynthetic bacteria are heterodimeric enzymes and, although they can also use reduced MV as a non-physiological electron donor, the bacterial enzymes use NADPH as the exclusive physiological electron donor and show no activity with reduced Fd as an electron donor (Vanoni and Curti 1999; Vanoni and Curti 2008).   Cyanobacteria contain, in addition to the Fd-dependent GOGAT described above, a heterodimeric GOGAT that is similar to the enzyme found in non-photosynthetic bacteria but which uses NADH instead of NADPH as the electron donor (Muro-Pastor et al. 2005; Navarro et al. 1995). The actual conversion of glutamine plus 2-oxoglutarate to glutamate, catalyzed by these heterodimeric bacterial GOGATs, is catalyzed by the larger α–subunit, with the β–subunit functioning solely to delivering electrons from NADPH to the α–subunit (Vanoni and Curti 1999; Vanoni and Curti 2008; Vanoni et al. 1996; Vanoni et al. 1998). The α–subunits of bacterial GOGATs exhibit significant similarities to the Fd-dependent GOGATs, in so far as prosthetic group content, mechanism, and the location of its two separated catalytic sites are concerned (Ravasio et al. 2002; van den Heuvel et al. 2002; Vanoni and Curti 1999; Vanoni and Curti 2008).

Although there is only a limited amount of structural information available for these enzymes, X-ray studies have revealed one striking difference between a Fd-

dependent enzyme and the α–subunit of a NADPH-dependent enzyme. The first

GOGAT tertiary structure to be solved (Binda et al. 2000) produced a 3.0 Å resolution

structure of the α–subunit of the NADPH-dependent GOGAT from the bacterium

Azospirillum brasilense (Protein Data Bank ID # 1EA0). Subsequently, a structure for

the Fd-dependent GOGAT from the cyanobacterium *Synechocystis* sp. PCC 6803, at 2.7

Å resolution (Ravasio et al.2002), became available (Protein Data Bank ID # 1LM1),

followed by two somewhat higher resolution structures (van den Heuvel et al. 2003) of

this cyanobacterial enzyme, one in a complex with the substrate 2-oxoglutarate (Protein

Data Bank ID # 1OFD) and one covalently modified by the inhibitor 6-diazo-5-oxo-1-

norleucine (Protein Data Bank ID # 1OFE). A comparison of the structures for the *A.*

brasilense and *Synechocystis* 6803 enzymes revealed that a loop, 27 amino acids long

(See Figure 1), is present in the *Synechocystis* enzyme but absent in the α-subunit of the

A. brasilense enzyme (Ravasio et al. 2002; van den Heuvel et al. 2003). An analysis of

amino acid sequences revealed that, while the amino acids comprising this loop are

present in all Fd-dependent GOGATs, they are not present in the α-subunits of

heterodimeric GOGATs from other non-photosynthetic bacteria (Hase et al. 2006; van

den Heuvel et al. 2002; van den Heuvel et al 2003; Vanoni amnd Curti 2008). The loop

is either 26 or 27 amino acids in length, depending on the species from which the enzyme

comes (Table 1 shows a comparison of the amino acid sequences for this loop in the Fd-

dependent GOGATs from several representative organisms). As shown in Table 2, the

loop is also absent in the α-subunits of the heterodimeric NADH-dependent GOGATs

found in cyanobacteria (Navarro et al. 1995; Navarro et al. 2000). The fact that an amino

acid "insert" of this length is present at this location in all Fd-dependent GOGATs, but

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absent from the α-subunits of all NADPH-dependent and NADH-dependent, heterodimeric GOGATs (Hase et al. 2006; Vanoni and Curti 2008), coupled with the reasonably close proximity of the loop to the prosthetic groups of the *Synechocystis* enzyme (See Figure 1) led to a proposal that this loop is involved in binding Fd to the enzyme at a position and in an orientation favorable for efficient electron transfer from reduced Fd to the [3Fe-4S] cluster of the enzyme (van den Heuvel et al. 2003; Vanoni and Curti 2008). To test this hypothesis, we have used site-directed mutagenesis to prepare a variant of *Synechocystis* 6803 GOGAT in which the putative Fd-binding loop has been deleted and have compared its properties to those of the wild-type enzyme.

Materials and Methods

      Synechocystis sp. PCC 6803 ferredoxin was expressed in *Eschericia coli* and purified as described previously (Xu et al., 2006). A variant of the wild-type GOGAT from the cyanobacterium *Synechocystis* sp. PCC 6803, in which six histidine residues were added to the enzyme at its C-terminus, was expressed in *E. coli*, using essentially the same procedure that was previously used to express the enzyme without a His-Tag (Navarro et al. 2000). The nucleotide sequence of the portion of the expression plasmid that coded for the His-Tagged version of wild-type GOGAT was confirmed by DNA sequencing in the Texas Tech University Biotechnology and Genomics Core Facility. After induction of GOGAT expression in the transformed *E. coli* cells and cell disruption using a French press, a filtered cell lysate containing only soluble proteins was prepared as described previously (Navarro et al. 2000). The filtered lysate, in 250 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-oxoglutarate plus 14.7 mM 2-

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mercaptoethanol, was loaded onto a 2 cm x 5 cm NTA Ni^{2+} affinity column (GE HealthCare) that had been equilibrated with this same buffer. The column was first washed with 10 column volumes of this same buffer, to which had been added 20 mM imidazole, to remove non-specifically bound proteins, and then the GOGAT was eluted with 5 column volumes of this same buffer, to which had been added 250 mM imidazole, The sample was concentrated using an Ultracel 100 kDa cut-off ultrafiltration membrane (Millipore) and buffer exchange carried out so that the sample was now in 30 mM Tricine-KOH buffer (pH 7.5), containing 1mM 2-oxoglutarate, 14.7 mM 2-mercaptoethanol and 20% (V/V) glycerol. The sample was then loaded onto a 2.5 cm x 20 cm affinity column, containing wild-type *Synechocystis* sp. PCC 6803 Fd covalently coupled to Sepharose 4B, that had been previously equilibrated using this buffer, and elution with this buffer was carried out to remove non-specifically bound protein. The sodium chloride concentration of the eluting buffer was then increased to 200 mM to elute GOGAT from the Fd-affinity column. The GOGAT-containing fractions were pooled, concentrated and subjected to gel filtration on a 1.5 cm x 80 cm Ultrogel AcA 34 column, using the same buffer used to elute the GOGAT from the Fd-affinity column. SDS-PAGE analysis indicated that all of the ferredoxin and wild-type GOGAT samples used for the experiments described below were approximately 95% pure. The presence of the C-terminal His-Tag was confirmed by Western blotting using an antibody (Invitrogen) directed against the six-histidine motif.

A variant of the His-tagged *Synechocystis* sp. PCC 6803 GOGAT missing the 27 amino acids of the putative Fd-binding loop, i.e. amino acid residues 907 through 933 (the numbering is based on designating the N-terminal cysteine of the mature form of the

wild-type GOGAT as amino acid 1) was prepared using site-directed mutagenesis. The

mutagenesis was carried out using the QuickChange site-directed mutagenesis kit

(Stratagene). PCR amplification was performed according to the manufacturer's

instructions, using 5' AAT TCC GGG GAA CCC CCA------- CAA AAT GGA GAC

ACG GCC 3' as the mutagenic primer in the forward direction and 5' GGC CGT GTC

TCC ATT TTG--------- TGG GGG TTC CCC GGA ATT 3' as the mutagenic primer in

the reverse direction. The nucleotide sequence of the coding region of the plasmid used

to express the loopless GOGAT variant was confirmed by DNA sequencing in the Core

Facility of the Texas Tech Center for Biotechnology and Genomics. Expression and

purification of the loopless GOGAT variant was carried out using the procedure

described above for wild-type GOGAT. The purity of the loopless GOGAT samples

used in the experiments described below was approximately 90%, based on SDS-PAGE

analysis, slightly less than that obtained for the wild-type enzyme. As was the case for

the wild-type enzyme, the presence of the C-terminal His-Tag was confirmed by Western

blotting.

      Absorbance spectra were measured using a Snimadzu Model UV-2401PC

spectrophotometer, at a spectral resolution of 0.5 nm. Circular dichroism (CD) spectra

were measured at a spectral resolution of 1.0 nm using an OLIS model DSM-10 UV-Vis

CD spectrophotometer. Protein concentration was estimated using the method of

Bradford (Bradford 1976), with bovine serum albumin used as a standard. FMN (Faeder

and Siegel 1973), non-heme iron (Massey, 1957), and acid-labile sulfide (Siegel et al.

1973) were measured using standard methods. Preparation of tryptic digests for MALDI-

TOF-TOF mass spectrometry and the mass spectrometry analyses themselves were

carried out as described previously (Kim et al. 2012; Srivastava et al. 2013). GOGAT activities, with either reduced Fd or reduced methyl viologen as an electron donor, were measured as described previously (Hirasawa et al. 1986). Kinetic data with reduced Fd serving as the electron donor was fitted to the Michaelis-Menten Equation using Prism fitting software. Isothermal titration calorimetry (ITC) was carried out, at 25 °C, using a MicroCal Model iTC$_{200}$ calorimeter. ITC binding and thermodynamic parameters were calculated using MicroCal software. The initial *in silico* docking model for the putative Fd/GOGAT complex was carried out using GRAMM-X (Tovchigrechko 2006) and the structures in the PDB for *Synechocystis* sp PCC 6803 Fd (1OFF) and *Synechocystis* sp PCC 6803 GOGAT (1LM1). The lowest energy docked orientation was refined using RosettaDock (Lyskov 2008). Surface electrostatic distributions were computed using the APBS plug-in for Pymol (Baker, 2001). All protein structures were rendered using Pymol (Schrodinger 2013).

Results

Even though DNA sequencing had confirmed that the GOGAT-coding portion of the plasmid used to direct synthesis of the loopless variant was indeed missing the 27 codons that encoded this loop, MALDI-TOF-TOF mass spectrometry analysis, accompanied by peptide mass fingerprint analysis, of tryptic digests of wild-type GOGAT and of its loopless variant were carried out to provide additional confirmation, at the protein level, that the 27 amino acids of the loop had indeed been deleted in the loopless variant. Two peptides were observed in the digest of the wild-type enzyme that clearly arise from the presence of the loop. These were: YLTLDDVDSEGNSPTLPHLH

GLQNGDTANSAIK, with a value for m/z of 3,492.69 Da; and SNSGEGGEDVVR, with m/z = 1,205.54 Da. MS/MS measurements of the two peptides confirmed the amino acid sequences deduced for these two m/z values. Neither peptide was observed in the mass spectrum of the loopless variant, providing evidence that the loop had been successfully deleted in the loopless variant.

It should also be mentioned that a comparison of the specific activities, with reduced Fd serving as the electron donor, measured for the wild-type GOGAT with the attached C-terminal His-Tag used in this study (see below) to the value reported previously for recombinant enzyme lacking the His-Tag (Navarro et al. 2000) were essentially identical for the two preparations, indicating the His-Tag does not interfere significantly with enzymatic activity with its physiological electron donor.   One difference that does exist between the two preparations is the significantly higher ratio of activity with Fd as the donor, compared to that with MV as the donor, observed in this study with the His-tagged enzyme when compared to the value observed for recombinant enzyme without a C-terminal His-tag. The value of this ratio is ca. 60, observed in this study, for GOGAT containing a His-tag and only ca. 6.5 in the case of enzyme without a His-tag (Florencio FJ and Navarro F; unpublished observations).

Before describing the prosthetic group content and catalytic properties of the loopless variant, it is worth mentioning that the variant is significantly less stable than the wild-type enzyme and the loss of activity encountered if additional purification steps were attempted prevented obtaining loopless variant preparations with purities significantly greater than 90%. It should also be mentioned that the Ni^{2+} affinity column was noticeably less effective in binding the loopless GOGAT variant than was the case

for the wild-type enzyme, despite the fact that DNA sequencing of the gene encoding this variant showed the presence of the expected six histidine codons at its 3'-end and the fact that Western blots of the loopless variant, using an antibody directed against the His-Tag, showed the six histidines to be present. This result suggested the possibility that deletion of the loop caused a conformational change significant enough to diminish access by the C-terminal His-Tag to the Ni^{2+} sites on the affinity matrix. However, CD spectra (not shown), in both the visible and near UV regions, of the loopless variant were very similar to those measured for the wild-type enzyme. Thus, although the presence of small conformational differences produced by deletion of the 27 amino acids of the loop cannot be ruled out, the CD data allow us to conclude that this deletion did not produce detectably significant changes in secondary structure.

If some differences in conformation between the wild-type enzyme and its loopless variant do in fact exist, even if they are relatively subtle ones, these might result in incomplete occupancy of one or both of the two prosthetic group binding sites. As shown in Table 3, the FMN content measured for the loopless variant was 0.75 mol FMN per mol of enzyme, was 85% of the 0.88 mol of FMN per mol of enzyme measured for wild-type enzyme (the theoretical value is 1.0). The loopless variant was slightly more deficient in iron-sulfur cluster content than was observed for FMN, containing only 76% of the acid-labile sulfide found in the wild-type enzyme in control measurements (i.e., 2.5 mol of sulfide per mol of enzyme in the loopless variant compared to 3.3 mol of sulfide per mol of enzyme for wild-type GOGAT). Both of these sulfide contents were significantly lower than the value of 4.0 mol of sulfide per mol of enzyme, based on the presence of one [3Fe-4S] cluster in the enzyme (Navarro et al. 2000; Ravasio et al. 2002;

van den Heuvel et al. 2002). As sulfide determinations often under-estimate the actual

value (Siiegel et al. 1973), attempts were also made to estimate the [3Fe-4S] cluster

content by measuring the amount of non-heme iron present in the two preparations.

However, the large amounts of adventitious iron present in both the wild-type enzyme

and its loopless variant made these measurements of little value.

Figure 2 shows the results of an ITC titration of the loopless GOGAT variant with

Fd, at low ionic strength. The data give a good fit for the formation of a 1:1 complex

between the two proteins, with K_d = 3.1 µM, a value that reflects an affinity that is

actually 2.7-fold greater than that observed in ITC titrations (not shown) carried out

under identical conditions with the wild-type GOGAT, where a value for K_d of 8.3 µM

was measured (A detailed ITC study of the enthalpy and entropy of complex formation

between Fd and several of its target enzymes is currently underway and the results of this

study will be reported in the future.). K_d values, calculated from the ITC binding

experiment data are summarized in Table 3.

In addition to being able to bind Fd, the loopless variant of GOGAT retains the

ability of the wild-type enzyme to utilize reduced Fd as an effective electron donor. As

can be seen from the kinetic parameters shown in Table 4, the loopless variant actually

exhibits a higher maximal velocity with reduced Fd serving as the electron donor than

does the wild-type enzyme. If one takes into account the fact that the loopless variant

may not have fully-occupied FMN and iron-sulfur cluster binding sites (see above), the

ratio of the Fd-dependent activity of the loopless variant compared to that of wild-type

GOGAT may even be greater than the 1.46-fold value calculated from the data in Table

4, if the activities of the wild-type enzyme and its loopless variant were to be compared

on either an equal FMN basis or an equal iron-sulfur cluster basis. The K_M values measured in this study for the wild-type recombinant GOGAT containing a C-terminal His-tag, are in reasonably good agreement with those reported earlier (Navarro et al, 2000) for recombinant *Synechocystis* sp. PCC 6803 GOGAT lacking a His-tag. While deletion of the loop had only a very small effect on the K_M for glutamine, the K_M values for both Fd and for 2-oxoglutarate we considerably higher for the loopless variant than for wild-type recombinant enzyme, increasing by a factor of 100 in the case of Fd and by a factor of slightly less than 80 in the case of 2-oxoglutarate. The loopless variant also exhibits higher activity with the non-physiological donor, reduced MV, than does the wild-type enzyme (Table 4).

Discussion

The kinetic and binding data presented above provides compelling evidence that the presence of the 27 amino acid loop that is unique to Fd-dependent GOGATs is not an absolute requirement for either Fd binding by the enzyme or for the ability of Fd to serve as an effective electron donor for the GOGAT-catalyzed reaction. In this context, it should be mentioned that cell-free extracts of a strain of *Synechocystis* sp. PCC 6803 in which the gene encoding its Fd-dependent GOGAT (i.e., *gstFglsF*) has been deleted are capable of catalyzing the GOGAT reaction with reduced Fd serving as the electron donor. This activity can be attributed to the presence of the enzyme encoded by the *gltB* gene, the NADH-dependent, heterodimeric GOGAT present in this cyanobacterium (Navarro et al 1995). The catalytic α-subunit of this NADH-dependent enzyme has been shown to be missing the loop found exclusively in Fd-dependent enzymes (Navarro et al. 1995). Thus, this observation provides additional support for the hypothesis that a GOGAT

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lacking this loop can support Fd-dependent activity and strengthens our conclusion that this loop is not involved in productive binding of Fd to the enzyme.

If the loop is not a part of the docking site for Fd, the obvious question raised is where the docking site might be. In an attempt to provide a possible answer to this question, we have undertaken an *in silico* modeling study designed to identify possible Fd-binding sites. Figure 3A shows the lowest energy solution to the search for a docking site and it is obvious that there is no contact between Fd and the 27 amino acids of the GOGAT loop. It should also be noted that, as expected from the fact that *Synechocystis* sp. PCC 6803 Fd possesses a net negative surface charge (Bottin and Lagoutte, 1992), the docking site for Fd on GOGAT exhibits a net positive charge and thus would produce favorable electrostatic interactions at the binding site. In contrast, as also shown in Figure 3, the region on GOGAT containing the 27 amino acids of the loop has a substantial net negative surface charge and binding Fd to this site would thus produce an electrostatically unfavorable interaction. Given how well "plant-type" ferredoxins are conserved (Hase at al. 2006), one would have expected that the loop would be well conserved if it did provide the binding site on GOGAT for Fd. However, as illustrated in Table 1, the loop is not particularly well conserved. Figure 3B shows an enlarged view of this region of the docking model, with the dashed line showing a possible pathway for electron transfer from the [2Fe-2S] cluster of Fd to the [3Fe-4S] cluster of GOGAT. The distance between the two clusters is ~14 Å in the rigid docking method used for this calculation. Structural differences in a more realistic model for the Fd/GOGAT complex that takes protein flexibility into account could decrease the distance between the two iron-sulfur clusters in this protein/protein complex significantly. Mechanistic studies

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have provided strong evidence for the hypothesis that electrons from reduced Fd enter the enzyme by first reducing its [3Fe-4S] cluster (Ravasio et al. 2002; van den Heuvel et al. 2002; van den Heuvel et al. 2003; Vanoni and Curti 2008), so the orientation of these two iron-sulfur clusters with respect to each other in any docking model is an important parameter.   It should be noted that, although not shown in Figure 3, the docking model predicts either electrostatic interactions or hydrogen bonds between four pairs of residues, i.e. Asp61 of Fd with Arg1162 of GOGAT, Asp67 of Fd with His 1144 of GOGAT, Gln62 of Fd with Asn1147 of GOGAT, and Glu71 of Fd with Gln467 of GOGAT. Experiments are being designed to use site-directed mutagenesis of both GOGAT and Fd to test these predictions.

Acknowledgement

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Table 1. Amino acid sequence alignment of eight ferredoxin-dependent glutamate synthases.

```
Synechocystis sp. PCC 6803    DVVRYLTLDDVDSEGNSPTLPHLHGLQ 27
Zea mays                      DPIRWNPLTDV-VDGYSPTLPHLKGLQ 26
Spinacia oleracea             DPIRWRPLTDV-VDGYSSTLPHLKGLQ 26
Arabidopsis thaliana          DPIRWKPLTDV-VDGYSPTLPHLKGLQ 26
Oryza sativa                  DPIRWSPLADV-EDGYSPTLPHLKGLQ 26
Porphyra purpurea             DPVRFKVLNDVNESGNSDLLPHLKGLR 27
Cyanidium caldarium           DSLRFTVLTDVDETGNSPSFPHLKGLK 27
Antithamnion sp.              DSTRFKSIQDLDTSGVSRTFSHLKGLK 27
                              *   *:   : *:    * *   : **:**
```

The number of amino acids present in the loop insert is shown in the column at the left. Positions where only a single amino acid is found are indicated by a * at the bottom of a column and positions where only conservative amino acid substitutions exist are indicated by a : at the bottom of the column.

Table 2. Amino acid sequences of cyanobacterial glutamate synthases. Sequences are shown in the region that contains the loop in Fd-dependent GOGATs. The first five sequences, shown in red, are for Fd-dependent enzymes. The next four sequences, shown in blue, are for the α-subunits of NADH-dependent GOGATs, all of which are lacking the loop (the missing amino acids of the loop are delineated by the dashed lines). Definitions for the species abbreviations used are shown below the table.

```
Syne. 6803   RLGAKSNSGEGGEDVVRYLTLDDVDSEGNSPTLPHLHGLQNGDTANSAIKQIASGRFGVTP
Cyano.7822   RIGGKSNSGEGGEDPTRFRVLDKIDETGSSAEFPHLKGLRKGDTASSAIKQIASGRFGVTP
Gloe. 7418   RIGGKSNSGEGGEDPIRYHVLDDVDAQGHSPLLPHLKGLKNGDTASSAIKQVASGRFGVTP
Nost.7120    RIGGKSNSGEGGEDPVRYKVLDDVDASGHSPTLPHLRGLRNGDTASSAIKQVASGRFGVTP
Gloe. 7421   RIGGKSNSGEGGEDPVRFKPLTDVLPDTTSPSLPGIVGLRNGDSASSAIKQVASGRFGVTP
Syne. 6803   RIGGKSNTGEGGEDPERFTWTND----------------QGDSKNSAIKQVASGRFGVTS
Gloecap      RIGGKSNTGEGGEDPERYTWTNS----------------QGDSKNSAIKQVASGRFGVTS
Leptolyn     RIGGKSNTGEGGEDPDRYTWSNE----------------QGDSKNSAIKQVASGRFGVTS
Cyano.7822   RIGGKSNTGEGGEDPDRYTWTNE----------------KGDSKNSAIKQVASGRFGVTS
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Gloe. 7418: *Gloeocapsa* sp. PCC 7428
Nost.7120 : *Nosto*c sp. PCC 7120
Gloe. 7421: *Gloeobacter violaceus* PCC 7421
Leptolyn: *Leptolyngbya boryana*
Cyano.7822: *Cyanothece* sp. PCC 7822

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Table 3. Prosthetic group content and Fd-binding affinities of wild-type GOGAT and its loopless variant.

GOGAT Form	Sulfide[a]	FMN[a]	K_d Ferredoxin (μM)
Wild-Type	3.3	0.88	8.3
Loopless	2.5	0.74	3.1

a: mol/mol of enzyme

Table 4. Kinetic parameters for wild-type GOGAT and its loopless variant.

GOGAT Form	Relative Fd-linked specific activity[a]	Relative MV-linked specific activity[b]	Fd K_M (µM)	Glutamine K_M (mM)	2-Oxoglutarate K_M (mM)
Wild-Type	100	100	0.60	1.27	0.042
Loopless	146	215	60	1.66	3.3

a:100% = 35.7 µmol of Fd oxidized per minute per mg of GOGAT
b:100% = 0.58 µmol of MV oxidized per minute per mg of GOGAT

Figure Legends

Figure 1. The region of *Synechocystis* sp. PCC 6803 GOGAT containing the putative Fd-binding loop. The peptide backbone is shown in gray and the proposed Fd-binding loop is shown in green. The [3Fe-4S] cluster is shown in red (iron) and yellow (sulfur) and the FMN is shown in a stick representation with the atoms colored according to type.

Figure 2. ITC titration of the loopless GOGAT variant with wild-type Fd. The central well of the microcalorimeter contained loopless GOGAT at a concentration of 160 µM in 10 mM potassium phosphate buffer (pH 7.5) containing 1 mM 2-oxoglutarate and 12.5 mM 2-mercaptoethanol in a volume of 250 µL. Fd, at a concentration of 660 µM in this same buffer, was added in 2 µL aliquots. The upper panel shows the actual calorimetric titration results and the lower panel shows the fit to a single binding isotherm for a 1:1 complex (The actual best fit value for the stoichiometry is 0.92 ± 0.13 Fd:1 GOGAT, with best-fit values of -4.17 ± 0.78 kcal/mol for ΔH and 10.8 ± 0.5 cal/mol/degree for ΔS)

Figure 3. An *in silico* docking model for the 1:1 complex between wild-type *Synechocystis* sp. PCC 6803 GOGAT and *Synechocystis* sp. PCC 6803 Fd. The peptide backbone of Fd is shown in beige and the atoms of its [2Fe-2S] cluster are shown as red and yellow spheres for the Fe and S, respectively. GOGAT is represented using spheres to represent surface amino acids. Atoms of the [3Fe-4S] cluster and of the isoalloxazine ring of FMN of GOGAT are shown as spheres, colored according to atom type. The surface charge distribution of GOGAT is depicted by showing positively-charged regions in blue and negatively-charged regions in red, with uncharged regions shown in white. A. The complete docking model. The 27 amino acids that have been deleted in the

loopless GOGAT variant, present in the upper left-hand portion of the figure, are highlighted using a stick representation to trace this portion of the peptide backbone. B. An enlarged view of the putative docking site for Fd on GOGAT and of the 27 amino acids of the loop. The dashed line represents a possible pathway for electron transfer from the reduced [2fe-2S] cluster of Fd to the oxidized [3Fe-4S] cluster of GOGAT.
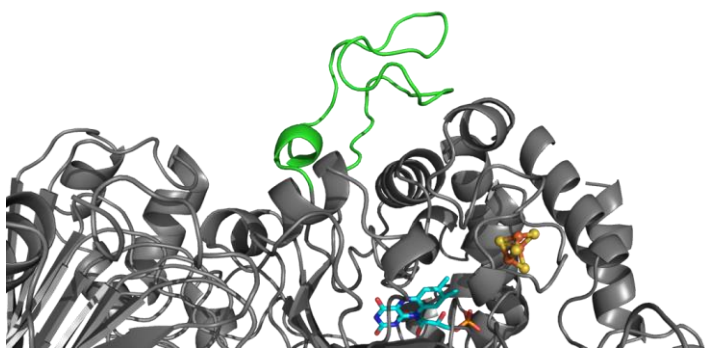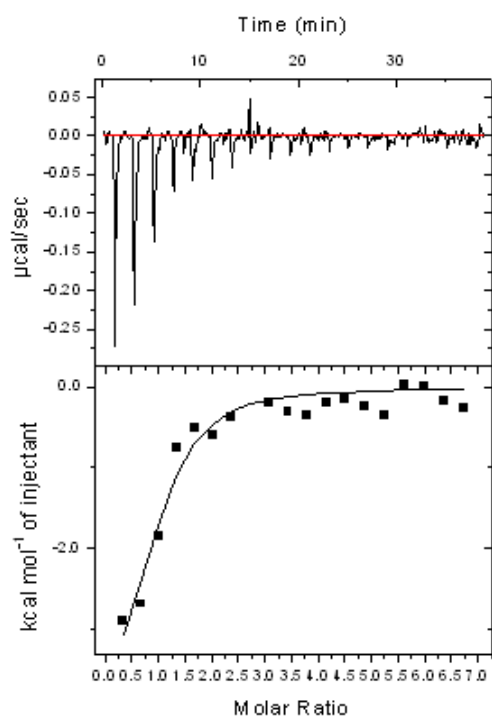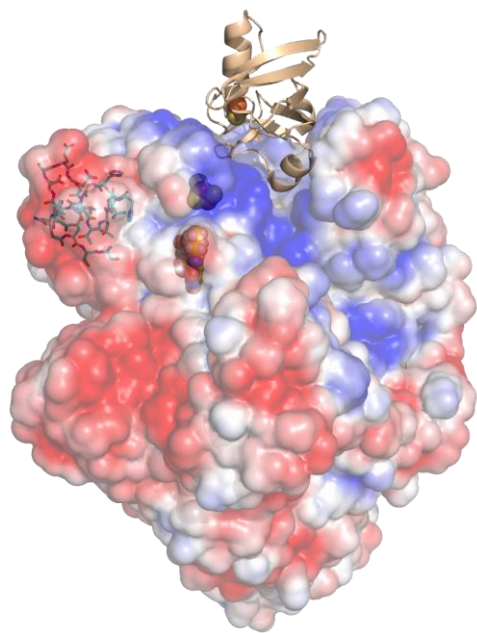
Figure 1

Figure 2.

Figure 3A

Figure 3B