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Review Article

Analysis of Functional Domains on Glutamate Synthase

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Abstract: Glutamate synthases (GOGAT) were analyzed to identify the functional binding domains of the substrate (glutamine) and cofactors (FMN, NAD(P)H, FAD, $[3Fe\text{-}4S]^{1+,0}$ and $[4Fe\text{-}4S]^{2+,1+}$ clusters and ferredoxin) on this enzyme. The published amino acid sequences of six different NAD(P)H-dependent GOGATs (NAD(P)H-GOGAT) and ten different ferredoxin-dependent GOGATs (Fd-GOGAT) were used for this analysis. The amino acid sequences of these sixteen GOGATs were compared with the amino acid sequences of aminotransferases for glutamine, flavoproteins for FMN, flavoproteins and pyridine-nucleotide-dependent enzymes for FAD and NAD(P)H, iron-sulfur proteins for $[3Fe\text{-}4S]^{1+,0}$ and $[4Fe\text{-}4S]^{2+,1+}$ clusters and ferredoxin-dependent enzymes for ferredoxin. It was determined that Fd-GOGAT has one domain each for glutamine, FMN and $[3Fe\text{-}4S]^{1+,0}$ cluster and two domains each for FAD and ferredoxin; the NADPH-GOGAT α subunit has the same domains as Fd-GOGAT except for the ferredoxin domains, and β subunit has one domain each for NADPH and FAD and two domains for two $[4Fe\text{-}4S]^{2+,1+}$clusters; NADH-GOGAT has the same domains as NADPH-GOGAT.

Key Words: Glutamate synthase, glutamine, FMN, FAD, NAD(P)H, iron-sulfur cluster, ferredoxin, binding domain.

Glutamat Sentaz Üzerindeki Fonksiyonel Bölgelerin Analizi

Özet: Glutamat sentazlar (GOGAT), bu enzim üzerindeki substrat (glutamin) ve kofaktörlerin (FMN, NAD(P)H, FAD, $[3Fe\text{-}4S]^{1+,0}$ ve $[4Fe\text{-}4S]^{2+,1+}$ kümeleri ve ferredoksin) fonksiyonel bağlanma bölgelerini belirlemek için analiz edildi. Altı farklı NAD(P)H'a bağımlı GOGAT (NAD(P)H-GOGAT)'ın ve on farklı ferredoksine bağımlı GOGAT (Fd-GOGAT)'ın yayınlanmış amino asit sıraları bu analiz için kullanıldı. Bu onaltı GOGAT'ın amino asit sırası glutamin için aminotransferazların, FMN için flavoproteinlerin, FAD ve NAD(P)H için flavoproteinler ve piridin nükleotidine bağımlı enzimlerin, $[3Fe\text{-}4S]^{1+,0}$ ve $[4Fe\text{-}4S]^{2+,1+}$ kümeleri için demir kükürt proteinlerin ve ferredoxin için ferredoksine bağımlı enzimlerin amino asit sıraları ile karşılaştırıldı. Fd-GOGAT'ın glutamin, FMN ve $[3Fe\text{-}4S]^{1+,0}$ kümesi için birer bölgeye ve FAD ve ferredoksin için ikişer bölgeye; NADPH-GOGAT α alt biriminin ferredoksin bölgeleri hariç Fd-GOGAT'daki gibi aynı bölgelere ve β alt biriminin NADPH ve FAD için birer bölgeye ve iki $[4Fe\text{-}4S]^{2+,1+}$ kümesi için iki bölgeye; NADH-GOGAT'ın NADPH-GOGAT'daki gibi aynı bölgelere sahip olduğu belirlendi.

Anahtar Sözcükler: Glutamat sentaz, glutamin, FMN, FAD, NAD(P)H, demir-kükürt kümesi, ferredoksin, bağlanma bölgesi

Introduction

Assimilation of ammonia into organic nitrogen is a result of the collaborative activity of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT) (1,2,3). GS catalyzes the incorporation of ammonia into the amide position of glutamate, producing glutamine. GOGAT catalyzes the reductive transfer of the amido group of glutamine to the α-keto position of 2-oxoglutarate, resulting in the formation of two molecules of glutamate (Fig.1).

The GOGAT enzyme found in several plants and bacteria occurs in three distinct forms; NADH-dependent GOGAT (NADH-GOGAT) (EC 1.4.1.14), NADPH-dependent GOGAT (NADPH-GOGAT) (EC 1.4.1.13) and ferredoxin-dependent GOGAT (Fd-GOGAT) (EC 1.4.7.1); these differ in molecular mass, subunit composition, enzyme kinetics, cofactor and antigenic specificity, and metabolic function (3,4,5,6). In higher plants, GOGAT occurs in two distinct isoforms; NADH-GOGAT and Fd-GOGAT. Fd-GOGAT is an iron-sulfur flavoprotein with a subunit molecular mass of 130-180 kDa that is generally considered to function as a monomer. Although NADH-GOGAT, like Fd-GOGAT, is also an iron-sulfur flavoprotein, this enzyme is found primarily in non-green tissues and it exists in the form of monomers with a native subunit mass of approximately 225-230 kDa. In bacteria, GOGAT occurs in two distinct isoforms; Fd-GOGAT and NADPH-GOGAT, which is composed of two subunits of approximately 135 kDa and 53 kDa for the large (α) and small (β) subunits, respectively (7).

Both plant and bacterial GOGATs, in addition to substrate (glutamine), require cofactors for intramolecular electron transfer in the reaction. It has been reported that Fd-GOGAT requires one ferredoxin, one $[3Fe\text{-}4S]^{1+,0}$ cluster, one FAD and one FMN; and that NADPH-GOGAT α subunit and NADH-GOGAT require one $[3Fe\text{-}4S]^{1+,0}$ cluster, one FAD and one FMN. In addition, NADPH-GOGAT β subunit and NADH-GOGAT require one NAD(P)H, one FAD and two $[4Fe\text{-}4S]^{2+,1+}$ clusters (3,4,8).

In order to determine the amino acid sequence of GOGAT enzymes, many cloning studies have been performed. Entire and partial amino acid sequences of GOGAT are reported from 16 different sources as shown in Fig.2. The 2089-amino acid sequence of *Saccharomyces cerevisiae* NADH-GOGAT was derived from its entire gene (9) and the 2093-amino acid sequence of alfalfa

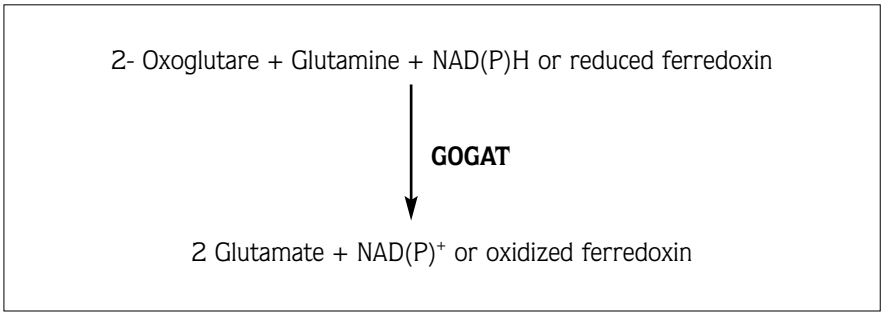

Figure 1. The reaction catalyzed by glutamate synthase (GOGAT).

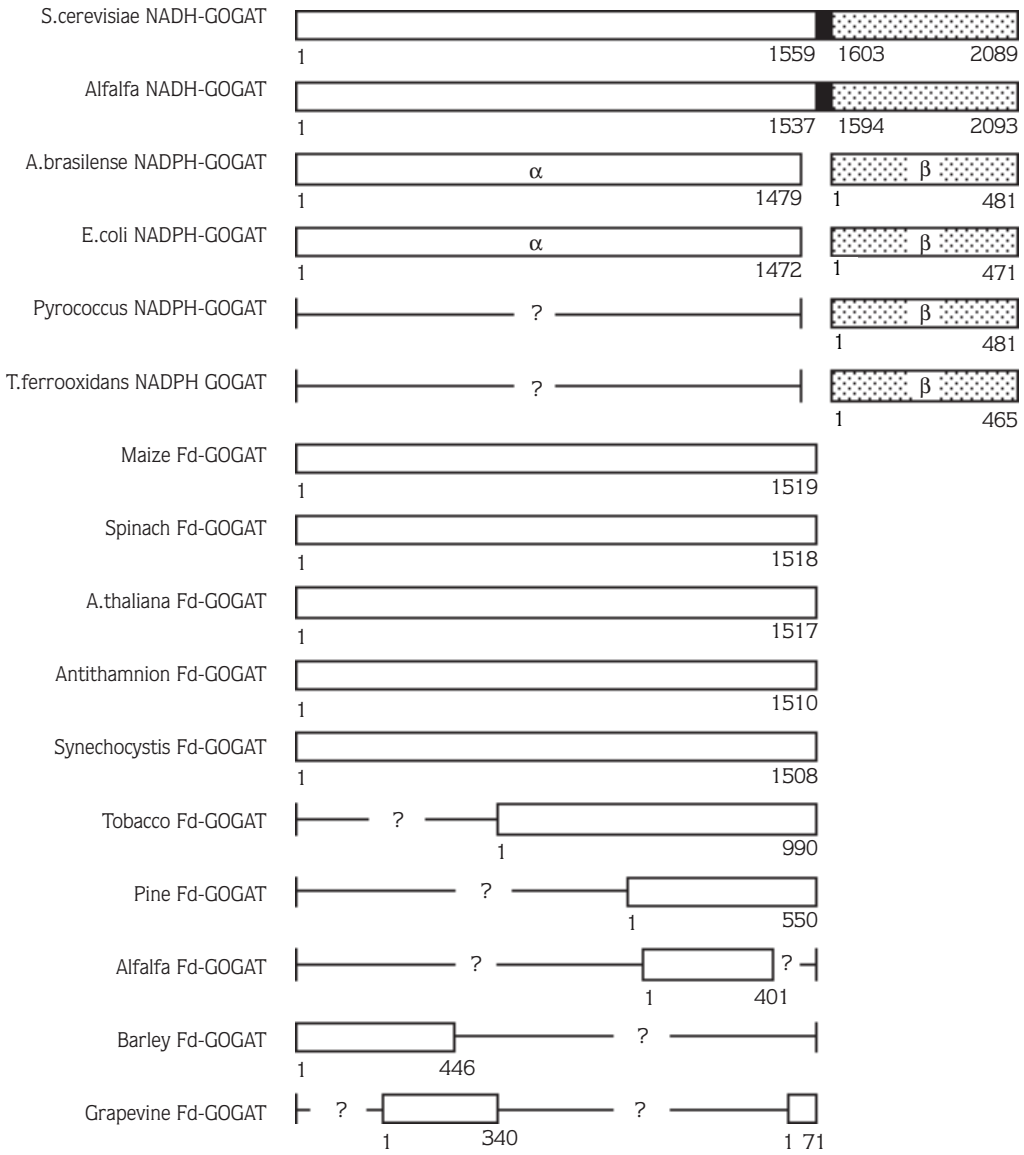

Figure 2. Diagrammatic comparison of the published GOGAT proteins from sixteen different sources. α and β indicate subunits. The darkened portion indicates a unique amino acid sequence that connects regions. Identically shaded areas indicate regions of sequence similarity. The ? portion indicates nonsequenced amino acid regions of GOGAT proteins.

NADH-GOGAT was derived from its entire complementary DNA (cDNA) (5); these entire proteins exist in the form of a monomer. The 1960-amino acid sequence of *Azospirillum brasilense* NADPH-GOGAT (10) and 1943-amino acid sequence of *Escherichia coli* NADPH-GOGAT (7) were derived from their entire genes; both of these entire proteins are composed of α and β subunits. The 481-amino acid sequence of *Pyrococcus* NADPH-GOGAT (4) and 465-amino acid sequence of *Thiobacillus ferrooxidans* NADPH-GOGAT (11) were derived from their partial genes; both of these partial genes encode only β subunits of the proteins as the amino acid sequences of the α subunits have not been determined. The 1519-amino acid sequence of maize Fd-GOGAT (6), 1518-amino acid sequence of spinach Fd-GOGAT (12) and 1517-amino acid sequence of *Arabidopsis thaliana* Fd-GOGAT (13) were derived from their entire cDNAs, and the 1510-amino acid sequence of *Antithamnion* Fd-GOGAT (14) and 1508-amino acid sequence of *Synechocystis* Fd-GOGAT (15) were derived from their entire genes; all of these five entire proteins exist in the form of a monomer and show homology with α subunits of NAD(P)H-GOGAT. The 990-amino acid sequence of tobacco Fd-GOGAT (16), 550-amino acid sequence of pine Fd-GOGAT (17), 401-amino acid sequence of alfalfa Fd-GOGAT (18), 446-amino acid sequence of barley Fd-GOGAT (19) and 340- and 71-amino acid sequences of grapevine Fd-GOGAT (20) were derived from their partial cDNAs; all of the partial regions of these five Fd-GOGAT proteins show high homology with the other Fd-GOGAT proteins. The other amino acid regions of these Fd-GOGATs have not been sequenced. A diagrammatic comparison of homologous regions found in *S. cerevisiae,* alfalfa, *A.brasilense, E.coli, Pyrococcus, T.ferrooxidans,* maize, spinach, *A.thaliana, Antithamnion, Synechocystis,* tobacco, pine, barley and grapevine GOGAT proteins is shown in Fig. 2.

This increased knowledge of the catalytic mechanism of GOGAT and of the number and types of flavin coenzymes, iron-sulfur clusters and ferredoxin in the bacterial and plant enzymes serves as a guide in the research of relevant regions on GOGAT. Therefore, in order to analyze functional binding domains for the substrate (glutamine) and cofactors (FMN, NAD(P)H, FAD, $[3Fe\text{-}4S]^{1+,0}$ and $[4Fe\text{-}4S]^{2+,1+}$ clusters and ferredoxin) on GOGAT, in this review, the known amino acid sequences of sixteen GOGATs were compared with amino acid sequences of aminotransferases for glutamine, flavoproteins for FMN, flavoproteins and pyridine-nucleotide-dependent enzymes for FAD and NAD(P)H, iron-sulfur proteins for $[3Fe\text{-}4S]^{1+,0}$ and $[4Fe\text{-}4S]^{2+,1+}$ clusters and ferredoxin-dependent enzymes for ferredoxin.

Analysis of Functional Domains on Glutamate Synthase

Glutamine Binding Domain

Glutamine amidotransferases are a family of enzymes that utilize the amide of glutamine for the biosynthesis of several amino acids, purine and pyrimidine nucleotides, folate and nicotinamide coenzymes, and the amino sugar glucosamine (21). Alignment of the amino acid sequences of different glutamine amidotransferases has permitted the identification of a glutamine amide transfer domain of approximately 180 amino acid residues at the NH_2 terminus of the amidotransferases because of high homology (21). In order to find essential amino acids

required for formation of the covalent glutaminyl intermediate, site-directed mutagenesis and affinity labeling of mutant enzymes were performed (21). The results of this study indicate that a Cys^{1}-Asp^{29}-His^{101} catalytic triad is involved in the glutamine amide transfer function of glutamine amidotransferases. The evidence suggests that His^{101} functions to increase the nucleophilicity of Cys^{1}, which is used to form a glutamine-enzyme covalent intermediate. Asp^{29} has a role subsequent to formation of the covalent intermediate (21). The Cys-Asp-His catalytic triad for glutamine binding domain is shown by using amino acid sequence alignment of three glutamine amidotransferases from *Escherichia coli* (E. coli Pur1) (22), *Bacillus subtilis* (B. subtilis Pur1) (23) and *Rhizobium leguminosarum* (R. leguminosarum NodM) (24) in (Fig. 3).

There has been only one study on *A.brasilense* NADPH-GOGAT to show the same catalytic triad on a GOGAT protein (25). In that study, it was shown that the pH dependence of the kinetic parameters of the glutamine-dependent reaction of the enzyme revealed the presence of ionizable groups with pK_a values between 6 and 10 involved in the binding of the substrates and in catalytic steps. The hypothesis that a group with pK_a between 8 and 10 is involved in the glutaminase segment of the glutamine-dependent GOGAT activity was supported by studies of the modification of the enzyme by a glutamine analog (6-diazo-5-oxo-L-norleucine) and a cysteine-directed reagent (iodoacetamide). Analyses of the kinetics of inactivation of the enzyme in the presence and absence of enzyme substrates and their analogs at different pH values demonstrated that the Cys residue at the N-terminal of the α subunit of *A.brasilense* NADPH-GOGAT is essential for the catalysis of the glutamine-dependent reaction and may be part of the Cys^{1}-Asp^{37}-His^{208} catalytic triad (25), as suggested for other glutamine amidotransferases (21) (Fig. 3).

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                  E.coli Pur1 1 CGIVG 5   28 QDAA   G 32   99 LAHNGNLT N 107  185 DP NG
              B.subtilis Pur1 1 CGVFG 5   28 QEGA   G 32   99 LAHNGNL VN 107  172 DP NG
         R.leguminosarum NodM 2 CGIVG 6   29 YD S  SG 33   95 VVHNGII EN 103  171  ARNG
                                *** *      **   **         * *    * *          * ***
      S.cerevisiae NADH-GOGAT 1 CGV G 4   36 SDGN   G 40  212 LVHSRFST N 220  350 D RNG
           Alfalfa NADH-GOGAT 1 CGV G 4   36 CEAN  TG 41  209 LIHSRFST N 217  352 D RNG
A.brasilense α NADPH-GOGAT 1 CGV G 4   36 ADGK  TG 41  206 IYHQRYST N 214  346 D RNG
      E.coli α NADPH-GOGAT 1 CG FG 4   36 ADGK  TG 41  200 LFHQRFST N 208  338 D RNG
            Maize Fd-GOGAT 1 CGV G 4   36 AD SD SG 41  207 IYHRRFST N 215  350 D RNG
          Spinach Fd-GOGAT 1 XGV G 4   36 SD ND SG 41  207 IYHRRYST N 215  350 D RNG
       A.thaliana Fd-GOGAT 1 CGV G 4   36 AD ND SG 41  207 IYHRRYST N 215  350 D RNG
     Antithamnion Fd-GOGAT 1 CGV G 4   36 AD NI SG 41  209 MYHRRFST N 217  351 D RNG
    Synechocystis Fd-GOGAT 1 CGV G 4   36 CE PNY G 41  208 LVHSRFST N 216  348 D RNG
           Barley Fd-GOGAT 1 XGV G 4   36 AD ND SG 41  207 IYHRRFST N 215  350 D RNG
        Grapevine Fd-GOGAT                            95 IYHRRYST N 103
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Figure 3. Analysis of glutamine binding domain on glutamate synthase. Amino acid sequences of eleven different GOGAT proteins were compared with three different glutamine amidotransferases. Bold residues indicate the Cys-Asp-His catalytic triad. X represents undetermined residue. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

In this review, in order to analyse the same catalytic triad on GOGAT proteins, homology between the NH_2-terminal regions of GOGATs from *S.cerevisiae* (9), alfalfa (5), *A.brasilense* (10), *E.coli* (7), maize (6), spinach (12), *A.thaliana* (13), *Antithamnion* (14), *Synechocystis* (15), barley (19) and grapevine (20) and glutamine amidotransferases from *E. coli* (22), *B. subtilis* (23) and *R. leguminosarum* (24) is illustrated by the alignment in (Fig. 3). As can be seen from the analysis, the same Cys-Asp-His catalytic triad is conserved in all GOGAT proteins. It appears that the glutamine binding domain on GOGAT is within the N-terminal ~350 residues of the α subunit of NADPH-GOGAT and of the single polypeptide chain of NADH-GOGAT and Fd-GOGAT proteins (Fig. 3).

In another study performed to determine the glutamine binding region on GOGAT, *A.brasilense* NADPH-GOGAT was subjected to limited proteolysis using tyrpsin and chymotrypsin, in the absence or presence of its substrates or their analogs (26). These analyses showed that the presence of the enzyme substrates or their analogs caused significant changes in the proteolytic patterns. The glutamine analog (L-methionine sulfone which binds presumably to the N-terminal region of the α subunit) altered the sensitivity to proteolysis of the sites of the α subunit. These results show that the N-terminal region of the α subunit of *A.brasilense* NADPH-GOGAT is important for glutamine binding (26), as mentioned in other studies carried out on glutamine amidotransferases (21) and GOGATs (25).

FMN Binding Domain

Glutamate synthases require FMN cofactor for intramolecular electron transfer in a glutamine-dependent reaction.No studies on GOGAT proteins aiming to identify FMN binding domains have been reported but there have been some studies on flavoproteins. One of these concerned alignment of the amino acid sequences of different flavoproteins which permitted the identification of a FMN binding domain of approximately 70 amino acid residues at flavoproteins in terms of high homology (27). This alignment of three flavoproteins from *S. cerevisiae* (S.cerevisiae b2) (28), *Hansenula anomala* (H.anomala b2) (27) and spinach (Spinach GO) (27) is shown in Fig. 4. In order to find essential amino acids required for binding of FMN, site-directed mutagenesis was performed on S.cerevisiae b2 (29). The results of these studies indicate that Lys-349 residue interacts with the isoalloxazine ring (i) of FMN and Asp-405 and Arg-413 residues interact with the ribityl side chain (r) of FMN (Fig. 4).

In order to analyse same the interaction of FMN on GOGAT proteins, in this review, the amino acid sequences of GOGAT proteins from *S.cerevisiae* (9), alfalfa (5,18), *A.brasilense* (10), *E.coli* (7), maize (6), spinach (12), *A.thaliana* (13), *Antithamnion* (14), *Synechocystis* (15), tobacco (16) and pine (17) were compared to the amino acid sequences of flavoproteins from S.cerevisiae b2 (28), H.anomala b2 (27) and Spinach GO (27) (Fig. 4). As can be seen from the analysis, the same Lys-Asp-Arg interaction triad is conserved in all GOGAT proteins. It appears that the FMN binding domain on GOGAT lies between the ~1021 and 1121 residues of NADH-GOGAT, the ~978 and 1077 residues of the α subunit of NADPH-GOGAT and between the ~1022 and 1108 residues of Fd-GOGAT proteins (Fig. 4).

S.cerevisiae b2	345	P	IVI	**K**	GVQRT	E	355	400	LKDK	LEV	FV		**D**GG	V	**R**RGTD	417
H.anomala b2	334	P	IVI	**K**	GVERK	E	344	386	LDQK	ID	TFV		**D**GG	V	**R**RGTD	403
Spinach GO	226	P	ILV	**K**	GVI	TAE	236	279	QGR	IPV	FL		**D**GG	V	**R**RGTD	293
		*	*	**	*	* **			*	*	*	*	***	*	*	* *	
S.cerevisiae NADH-GOGAT	1021	PRAGIS	V	**K**	LVS	E	1032	1090	LR	RNV	V	VQT	**D**G	QL	**R**TGFD	1107
Alfalfa NADH-GOGAT	1035	PAARIS	V	**K**	LVS	E	1046	1104	LRGR	T	T	LQT	**D**G	QL	**K**TGRD	1121
A.brasilense α NADPH-GOGAT	992	PDAKVT	V	**K**	LVSR		1003	1060	LRHR	VRLR			**T**DGG	L	**K**TGRD	1077
E.coli α NADPH-GOGAT	978	PKAMIS	V	**K**	LVS	E	989	1047	LRHK	IRLQ	V		**D**GG	L	**K**TGVD	1064
Maize Fd-GOGAT	1022	PKAKVS	V	**K**	LVS	E	1033	1091	LRER	VVLR	V		**D**GG		**F**RSGQD	1108
Spinach Fd-GOGAT	1022	PKAKVS	V	**K**	LV	AE	1033	1091	LRER	VILR	V		**D**GG	L	**K**CGVD	1108
A.thaliana Fd-GOGAT	1022	PNAKVS	V	**K**	LV	AE	1033	1091	LRER	VILR	V		**D**GG	L	**K**SGVD	1108
Antithamnion Fd-GOGAT	1022	PDAQVS	V	**K**	LV	A	1032	1091	LREK	VILR	V		**D**GG	L	**R**TGKD	1108
Synechocystis Fd-GOGAT	999	EARINV	K	**K**	LVS	E	1009	1066	LRSR	IVVE			TDG	QM	**K**TGRD	1083
Tobacco Fd-GOGAT	490	PRAKVS	V	**K**	LV	AE	501	558	LRER	VVLR	V		**D**GG		**F**KSGFD	575
Pine Fd-GOGAT	55	PMAKVS	V	**K**	LV	AE	66	124	LRER	VVLR	V		**D**G	D	**F**KSGVD	141
Alfalfa Fd-GOGAT	23	PKAKVS	V	**K**	LV	AE	34	92	LRER	VILR	V		**D**GG		**F**RSGVD	109
				i									**r**		**r**		

Figure 4. Analysis of FMN binding domain on glutamate synthase. Amino acid sequences of twelve different GOGAT proteins were compared with three different flavoproteins. Bold residues indicate interaction with isoalloxazine ring (i) and ribityl side chain (r) of FMN. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y), (S, T) and (K, R).

NAD(P)H and FAD Binding Domains

Glutamate synthases require NAD(P)H and FAD cofactors for intramolecular electron transfer in the reaction. No studies on GOGAT proteins to identify NAD(P)H and FAD binding domains have been reported but there some studies on flavoproteins and pyridine nucleotide-dependent flavoenzymes. One of these concerned alignment of the amino acid sequences of a number of flavoproteins and pyridine nucleotide-dependent flavoenzymes (30). These alignments are shown for three NAD(P)H-dependent flavoenzymes from *Trypanosoma* (Trypanosoma TR), *Saccharomyces* (Saccharomyces LD) and *E.coli* (E.coli LD) in Fig. 5 and three FAD-dependent flavoenzymes from Human (Human GR), *E.coli* (E.coli NR) and *Pseudomonas* (Pseudomonas LD) are shown in Fig. 6. From these analyses it became clear that a glycine-rich domain in terms of high homology is important for binding of NAD(P)H and FAD. In other research, site-directed mutagenesis, molecular modelling and atomic structure have been used to identify the function of the glycine-rich domain (31,32,33). It was determined that the glycine-rich domain interacts with the adenosine pyrophosphoryl moiety of NAD(P)H and FAD.

In order to analyse the similar interaction of NAD(P)H and FAD on GOGAT proteins, in this review, the amino acid sequences of GOGAT proteins were compared to the amino acid sequences of the flavoenzymes. For the NAD(P)H binding region, six different GOGATs from *S.cerevisiae* (9), alfalfa (5), *A.brasilense* (10), *E.coli* (7), *Pyrococcus* (4) and *T. ferrooxidans* (11) were compared to Trypanosoma TR, Saccharomyces LD and E.coli LD flavoenzymes (30) (Fig. 5). As can be seen from the analysis, a similar glycine-rich domain is conserved in all GOGAT proteins. It appears that the NAD(P)H binding domain on GOGAT lies between the ~1728 and 1775 residues of NADH-GOGAT and between the ~149 and 205 residues of the β subunit of NADPH-GOGAT proteins (Fig. 5). One study which aimed to determine the NADPH binding

region on GOGAT supports these findings (26). In this study, *A.brasilense* NADPH-GOGAT was subjected to limited proteolysis using trypsin and chymotrypsin, in the absence or presence of its substrates or their analogs. These analyses showed that the β subunit of *A.brasilense* NADPH-GOGAT is important for NADPH binding (26), as mentioned in this review. For the FAD binding region, twelve different GOGATs from *S.cerevisiae* (9), alfalfa (5,18), *A.brasilense* (10), *E.coli* (7), maize (6), spinach (12), *A.thaliana* (13), *Antithamnion* (14), *Synechocystis* (15), tobacco (16) and pine (17) were compared with Human GR, E.coli NR and Pseudomonas LD flavoenzymes (30) (Fig. 6). As can be seen from the analysis, a similar glycine-rich domain is conserved in all GOGAT proteins. It appears that the FAD binding domain on GOGAT lies between the ~1388 and 1444 residues of NADH-GOGAT, the ~1344 and 1396 residues of the α subunit of NADPH-GOGAT and between the ~1388 and 1428 residues of Fd-GOGAT proteins (Fig. 6).

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                Tyrpanosoma TR  147 V G G  G FIS  VEFAG IFN AY KP  V  GGKVT   L  CY      174
              Saccharomyces LD  145 IIG G  GI     I   G    L  E    M  G SV YSRLGSKVTVV E  170
                    E.coli LD  145 VMG G  GI     I   G    L  E    M  G TV YHALGSQIDVV E  170
                                   *** *  **     *   *    * ***   *  ** * *   *     * *
    S.cerevisiae NADH-GOGAT 1731 VIGSGPAGLACADMLNRAGHTVTV YERSCR  CGGLLMY   GIPNMKL D 1775
         Alfalfa NADH-GOGAT 1728 IVGSGPSGLAAADQLNKMGHIVTV FERADRI  GGLMMY   GVPNMK TD 1772
 A.brasilense β NADPH-GOGAT  152 VIGAGPAGLAAAEELRAKGYEVHV YDRYDRM  GGLLVY   GIPGFKL E  196
       E.coli β NADPH-GOGAT  151 IIGAGPAGLACADVLTRNGVKA VCFDRHPEI  GGLLTF   GIPAFKL E  195
   Pyrococcus β NADPH-GOGAT  161 IIGAGPAGLTAAGELAKLGYDVTI YE ALH EPGGVLMY   GIPEFRLP   205
T.ferrooxidan β NADPH-GOGAT  149 VVGGGPAGLGCADILNRAGTEVVV FDRYPAV  GGLLTF   GIPSFKL E  193
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Figure 5. Analysis of NAD(P)H binding domain on glutamate synthase. Amino acid sequences of six different GOGAT proteins were compared with three different NAD(P)H-dependent flavoenzymes including a glycine-rich domain. Bold residues indicate interaction with the adenosine pyrophosphoryl moiety of NAD(P)H. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

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                  Human GR   16 VI G  G  G     SGG     LA S ARR     A  A E LG  ARAAVV  E   41
                   E.coli NR   14 VV G  G  G  L L G   LE  A   A       G  ALKNLG I ETHVI  E   39
                Pseudomonas LD 15 II G  G  G   P  GG Y   VA   AIR     A  G Q LG I PTVLV  E   40
                                  ** *  *  *    ****  *  ** * * *     *  **   * **   **  *
    S.cerevisiae NADH-GOGAT 1388 VVE RKGNNAF EYMTGGRA AIV LS  Q MESLNAFSG AT GGIA YCL TSD 1432
         Alfalfa NADH-GOGAT 1404 VVEGV GDHG CEYMTGGTVV VLGKT G RNF   AA GM S GGIA Y VL  D 1444
 A.brasilense α NADPH-GOGAT 1356 VVEGC GSNG CEYMTGGTAV ILGR VGD NF   AA GM T GGMA Y V Y D 1396
       E.coli α NADPH-GOGAT 1344 VVEGI GDNG CEYMTGGIV CILGKT GV NF   GA GM T GGFA Y VL  D 1384
             Maize Fd-GOGAT 1388 VVEGT GDH CCEYMTGGCVV VLGK AG RN  V AA GM T GGLA Y IL  D 1428
           Spinach Fd-GOGAT 1388 VVEGT GDH CCEYMTGGCVV ILGK VG RN  V AA GM T GGLA Y IL  D 1428
        A.thaliana Fd-GOGAT 1388 VVEGT GDH CCEYMTGGCVV VLGK VG RN  V AA GM T GGLA Y LL  D 1428
      Antithamnion Fd-GOGAT 1388 VVEGV GDHA CEYMTGGLIV VLG TFG RN  I GA GM T GGIA YFL   D 1428
     Synechocystis Fd-GOGAT 1364 VVEAV GDHG CEYMTGGKVV VLGQT G RNF   AA GM S GGVA Y I F D 1404
          Tobacco Fd-GOGAT  856 VVEGT GDH CCEYMTGGCVV VLGK VG RN  V AA GM T GGL TY IL  D  896
             Pine Fd-GOGAT  421 IVEGT GDH CCEYMTGGCVV VLGK VG RN  V AA GM T GGLA Y LL  D  461
          Alfalfa Fd-GOGAT  389 VVEGA GDH CCEYI                                          401
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Figure 6. Analysis of FAD binding domain on glutamate synthase. Amino acid sequences of twelve different GOGAT proteins were compared with three different FAD-dependent flavoenzymes including a glycine-rich domain. Bold residues indicate interaction with the adenosine pyrophosphoryl moiety of FAD. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

For the FAD binding region, in addition to the glycine-rich domain, second sequence similarity has been determined on several FAD binding enzymes (34). This conserved region is shown on three FAD binding enzymes from *Azobacter vinelandii* (A. vinelandii DD), *E.coli* (E. coli TR) and humans (Human GR) (Fig. 7). The structural basis for this conserved region is clear from their three-dimensional structure. For all three structures it is known that the conserved Asp (Fig. 7) forms hydrogen bonds with the O-3 group of the ribityl chain of the flavin moiety of FAD (34). In order to analyse the similar interaction of FAD on GOGAT proteins, the amino acid sequences of GOGAT proteins were compared to the amino acid sequences of the FAD binding enzymes. Fourteen different GOGATs from *S.cerevisiae* (9), alfalfa (5,18), *A.brasilense* (10), *E.coli* (7), *Pyrococcus* (4),*T. ferrooxidans* (11), maize (6), spinach (12), *A. thaliana* (13), *Antithamnion* (14), *Synechocystis* (15), tobacco (16) and pine (17) were compared to A. vinelandii DD, E. coli TR and Human GR FAD binding enzymes (34) (Fig. 7). As can be seen from the analysis, a similar domain having Asp is conserved in all GOGATs and two different FAD binding domains are localized on GOGAT proteins. It appears that the first FAD binding domain on GOGAT lies between the ~1313 and 1339 residues of NADH-GOGAT, the ~1270 and 1291 residues of the α subunit of NADPH-GOGAT and between the ~1313 and 1329 residues of Fd-GOGAT proteins (Fig. 7). The second FAD binding domain lies between the ~2024 and 2037 residues of NADH-GOGAT and between the ~427 and 445 residues of the β subunit of NADPH-GOGAT proteins (Fig. 7).

```
          S.cerevisiae NADH-GOGAT 1313  S     GIT  FI  LNGD 1322
               Alfalfa NADH-GOGAT 1330     P  GITL  E  LEGD 1339
     A.brasilense α NADPH-GOGAT 1282   Q    GIKL  E  VMGD 1291
           E.coli α NADPH-GOGAT 1270       GGVEL Y   LTGD 1279
                 Maize Fd-GOGAT 1313 T   P  GMNI  R  LVGE 1323
               Spinach Fd-GOGAT 1313 T   P  GMNI  R  LVGE 1323
            A.thaliana Fd-GOGAT 1314     P  GMNI  R  LIGE 1323
          Antithamnion Fd-GOGAT 1320      K GIHL Y   LKGE 1329
         Synechocystis Fd-GOGAT 1290      K GMTL  E  LEGD 1299
               Tobacco Fd-GOGAT  781 T   P  GMNI  R  LIGE  791
                  Pine Fd-GOGAT  346 T   P  GMNI  R  LVGE  356
               Alfalfa Fd-GOGAT  314 TS     GMNI  R  LVGE  324
                                     *** ** *  * *   * **
                A.vinelandii DD  308 TS VP  G  V Y A I GD  318
                      E.coli TR  276 TS IP  G  V F AA  GD  286
                       Human GR  321 T NV K G  I Y A V GD  331
                                     ****** *  * * **  **
          S.cerevisiae NADH-GOGAT 2026  S I  DGGK TF A  CGD 2037
               Alfalfa NADH-GOGAT 2024 TS V  DG  V F AA  GD 2034
     A.brasilense β NADPH-GOGAT  433 T NM  DG  V F AA  GD  443
           E.coli β NADPH-GOGAT  433 TSN PK    I F AG  GD  443
        Pyrococcus β NADPH-GOGAT  435 TS IP  G  V F AG  GD  445
   T.ferrooxidans β NADPH-GOGAT  427 TSN PR    I F AG  GD  437
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Figure 7. Analysis of two FAD binding domains on glutamate synthase. Amino acid sequences of fourteen different GOGAT proteins were compared with three different FAD binding enzymes. Bold residues indicate interaction with ribityl moiety of FAD. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

[3Fe-4S]$^{1+,0}$ and [4Fe-4S]$^{2+,1+}$ Cluster Binding Domains

Glutamate synthases require [3Fe-4S]$^{1+,0}$ and [4Fe-4S]$^{2+,1+}$ clusters for intramolecular electron transfer in the reaction. These iron-sulfur ([Fe-S]) clusters are ligated to the polypeptide backbone primarily via cysteine residues (35). No studies on GOGAT proteins to identify [3Fe-4S]$^{1+,0}$ and [4Fe-4S]$^{2+,1+}$ cluster binding domains, a study have been reported but there have been some studies on iron-sulfur proteins. Electron paramagnetic resonance, magnetic circular dichroism, mass spectroscopy, resonance Raman data, site-specific mutagenesis, and comparison of the arrangement of cysteine residues of proteins involving iron-sulfur clusters reveal that there is one region for [3Fe-4S]$^{1+,0}$ and two regions for two [4Fe-4S]$^{2+,1+}$ clusters (35-38). These cysteine alignments are shown for the formation of a [3Fe-4S]$^{1+,0}$ cluster in three iron-sulfur proteins from *E. coli* (E.coli FrdB), Beef Heart (B.Heart SdhIp) and *Peptococcus aerogenes* (P.aerogenes 8Fe Fd) (36) (Fig. 8), and for the formation of two [4Fe-4S]$^{2+,1+}$ clusters in three iron-sulfur proteins from *Azotobacter vinelandii* (A.vinelandii FdIII), *Francisella tularensis* (F.tularensis Fd) and *E. coli* (E.coli ORF86) (35) (Fig. 9). From all analyses it became clear that only 3 cysteinyl residues are required to coordinate a [3Fe-4S]$^{1+,0}$ cluster and 4 cysteinyl residues are required for a [4Fe-4S]$^{2+,1+}$ cluster.

In order to analyse cysteine-rich domains required in the formation of [3Fe-4S]$^{1+,0}$ and [4Fe-4S]$^{2+,1+}$ clusters on GOGAT proteins, the amino acid sequences of GOGAT proteins were compared to the amino acid sequences of the iron-sulfur proteins. For the [3Fe-4S]$^{1+,0}$ cluster binding region, twelve different GOGATs from *S.cerevisiae* (9), alfalfa (5,18), *A.brasilense* (10), *E.coli* (7), maize (6), spinach (12), *A.thaliana* (13), *Antithamnion* (14), *Synechocystis*

```
                E.coli FrdB  204 C TFVGYC SEV   C  214
              B.Heart SdhIp  214 C HTIMNC TE   TC  224
          P.aerogenes 8Fe Fd   34 CIDC GSCAS V   C   44
                                  **  *  * * *  **
     S.cerevisiae NADH-GOGAT 1131 CVM LRRCH  LN SC 1142
           Alfalfa NADH-GOGAT 1145 CIM MRKCH K N TC 1156
A.brasilense α NADPH-GOGAT 1102 CIM VRQCHS  N TC 1113
       E.coli α NADPH-GOGAT 1088 C KYLRICH  LNN C 1099
             Maize Fd-GOGAT 1132 CVMA RICHT  NN C 1143
           Spinach Fd-GOGAT 1132 CVMA RICHT  NN C 1143
         A.thaliana Fd-GOGAT 1132 CVMA RICHT  NN C 1143
       Antithamnion Fd-GOGAT 1132 CVMA RVCHT  NN C 1143
      Synechocystis Fd-GOGAT 1108 CIM MRACH  LN TC 1119
           Tobacco Fd-GOGAT  600 CVMA RICHT  NN C  611
              Pine Fd-GOGAT  165 CIMA RICHT  NN C  176
            Alfalfa Fd-GOGAT  133 CVMA RICHT  NN C  144
```

Figure 8. Analysis of [3Fe-4S]$^{1+0.}$ cluster binding domain on glutamate synthase. Amino acid sequences of twelve different GOGAT proteins were compared with three different iron-sulfur proteins. Bold residues indicate interaction in formation of [3Fe-4S]$^{1+,0}$ cluster. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

(15), tobacco (16) and pine (17) were compared to E.coli FrdB, B.Heart SdhIp and P.aerogenes 8Fe Fd iron-sulfur proteins (36) (Fig. 8). As can be seen from the analysis, a similar cysteine-rich domain is conserved in all GOGAT proteins. It appears that the $[3Fe\text{-}4S]^{1+,0}$ cluster binding domain on GOGAT lies between the ~1131 and 1156 residues of NADH-GOGAT, the ~1088 and 1113 residues of the α subunit of NADPH-GOGAT and between the ~1132 and 1143 residues of Fd-GOGAT proteins (Fig. 8). For two $[4Fe\text{-}4S]^{2+,1+}$ cluster binding regions, six different GOGATs from *S.cerevisiae* (9), alfalfa (5), *A.brasilense* (10), *E.coli* (7), *Pyrococcus* (4) and *T.ferrooxidans* (11) were compared to A.vinelandii FdIII, F.tularensis Fd and E.coli ORF86 iron-sulfur proteins (35) (Fig. 9). As can be seen from the analysis, similar cysteine-rich domains are conserved in all GOGAT proteins. It appears that two $[4Fe\text{-}4S]^{2+,1+}$ cluster binding domains on GOGAT lie between the ~1624 and 1643 and ~1675 and 1691 residues of NADH-GOGAT and between the ~44 and 59 residues and ~94 and 113 residues of the β subunit of NADPH-GOGAT proteins (Fig. 9).

Ferredoxin Binding Domains

Electron transfer reactions are known to play fundamental roles in a wide variety of biological systems, and the mechanisms of these reactions have been the subject of intense study for more than two decades (39). Ferredoxin (Fd) is a central protein for transferring electrons from the photosynthetic chain to several Fd-dependent enzymes. Fd forms specific electrostatic complexes with Fd-dependent enzymes; one of these is GOGAT (40). In order to show how the complexes are formed, various studies have utilised site-directed mutagenesis, potential distribution and dipole moment orientation, chemical modifications, cross-linking, crystallographic and isothermal titration calorimetry methods (39-48). It has been stated that stabilization of the functional complexes between the two proteins is the result of the electrostatic interaction between acidic (negatively charged, such as aspartate [D] and glutamate [E]) side chains on a Fd and basic (positively charged, such as lysine [K] and arginine [R]) side chains on a Fd-dependent enzyme. It is of interest, therefore, to determine which basic residues in Fd-dependent enzymes are responsible for the recognition of Fd. There has been no any specific study of basic residues on GOGAT proteins but there have been some studies on another

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          A.vinelandii FdIII     8 CI NCDV    CEPE   C    18    37 CTE CVGHYDEPQCQQVC    53
             F.tuleransis Fd     9 CI NCDI    CEPE   C    19    38 CTE CVGHFEESQCTKVC    54
              E.coli ORF-086     8 CI NCDM    CEPE   C    18    37 CTE CVGHYETPTCQKVC    53
                                   ** **      *  *   *             *   * *  *   *   *
     S.cerevisiae NADH-GOGAT 1629 CMD CGTPF  CLSD TGC 1643 1680  GRVCPAP     CEGAC 1691
          Alfalfa NADH-GOGAT 1624 CMD CGTPF  CHQENSGC 1639 1675  GRVCPA P    CEGSC 1686
   A.brasilense β NADPH-GOGAT   47 C SQCGVPF  C Q VH C   59    94 CGRICP Q DRL CEGNC  108
        E.coli β NADPH-GOGAT   48 C R  AANPY C  EWK C   59    94 CGRVCP Q DRL CEGSC  108
     Pyrococcus β NADPH-GOGAT   44 CL QCPYEYAPCI   KGC   58   100  GRVCP QED  QCEMNC  113
T.ferrooxidans β NADPH-GOGAT   45 CLH CGNPY  C  EWK C   57    96 CGRICP Q DRL CEGAC  106
```

Figure 9. Analysis of two $[4Fe\text{-}4S]^{2+,1+}$ cluster binding domains on glutamate synthase. Amino acid sequences of six different GOGAT proteins were compared with three different iron-sulfur proteins. Bold residues indicate interaction in formation of $[4Fe\text{-}4S]^{2+,1+}$ clusters. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

Fd-dependent enzyme: spinach ferredoxin: NADP+ oxidoreductase (FNR). In one study, the potential distribution and dipole moment orientation of Fd suggested that the negative domain of Fd with D-65 and D-66 matches the positive domain of spinach FNR with K-33, K-35, K-91 and R-93 (43). In two other studies, it was determined through site-directed mutagenesis and isothermal titration calorimetry that K-88 of spinach FNR is required for the interaction with E-92 of Fd (44,46). These results suggest that K-33, K-35, K-88, K-91 and R-93 on spinach FNR are key residues in the binding to Fd, as shown in (Fig. 10). Since there have been no reports of sequence aligment for Fd binding region(s) on Fd-dependent enzymes, in this review six different Fd-dependent enzymes were compared for lysine and arginine-rich regions. It was found that there are two regions having high homology for K and R residues on Fd-dependent enzymes (Fig. 10).

In order to analyse lysine and arginine-rich domain(s) required in reaction on GOGAT proteins, the amino acid sequences of GOGAT proteins were compared to the amino acid sequences of the Fd-dependent enzymes. For Fd binding region(s), seven different GOGATs from maize (6), spinach (12), *A.thaliana* (13), *Antithamnion* (14), *Synechocystis* (15), tobacco (16) and pine (17) were compared to three different FNRs from spinach (Spinach FNR) (49), rice (Rice FNR) (50) and *Mesembryanthemum crystallinum* (M.crystallinum FNR) (51) and three different nitrite reductases (NiRs) from maize (Maize NiR) (52), spinach (Spinach NiR) (53) and rice (Rice NiR) (54) (Fig. 10). As can be seen from the analysis, two similar lysine and arginine-rich domains are conserved in all GOGAT proteins. It appears that two Fd binding domains on GOGAT lie between the ~961 and 990 and the ~1436 and 1446 residues of Fd-GOGAT proteins (Fig. 10). The presence of two Fd binding regions is also supported by the experiments of two previous studies (40,41).

```
          Spinach FNR  15 KV   EKHS       K KMEE   GITVNKFKP K  35   85 K NGK PH KL   R   93
            Rice FNR  16 K K  EKIS       K K HD  EGVVTNKYRP K  36   86 K NGK PH KL   R   94
     M.crystalium FNR  14 KV   EKHS       K KMEE   GVIVNKYKP K  34   84 K NGK PH KL   R   92
           Maize NiR  85 KL  T K  DDVDV  RLK   WL GLFH R R  K 105  305 RGN R  Q K T  R  312
          Spinach NiR 112 K HN  K  DDIDV  RLK   WL GLFH R R  K 132  334 RGN R  Q K C  R  341
            Rice NiR 114 KL  S K  EDIDV  RLK   WL GLFH R R  K 134  336 RGN R  Q K T  R  343
                        ** *  *  *  *  * ** ** *    * *  *        * * *   **   *
       Maize Fd-GOGAT 961 KIAQGAKPGEGGQLPGK KVSAYI A    RLRNSK 990 1436 KVN K EIVKM Q R 1446
      Spinach Fd-GOGAT 961 KIAQGAKPGEGGQLPGK KVSAYI A    RLRNSK 990 1436 KVN K EIVKI Q R 1446
  A.thaliana Fd-GOGAT 961 KVAQGAKPGEGGQLPGK KVSAYI A    RLRSSK 990 1436 KIN R ETVKI Q R 1446
Antithamnion Fd-GOGAT 961 KIAQGAKPGEGGQLPGK KVSPYI A EL R  NCK 990 1436 KLN  TEIVK AQ R 1446
Synechocystis Fd-GOGAT 937 KMAQGAKPGEGGQLPGK KVYPWI A    KVRHS  965 1412 RCN  SAMV  GLEK 1422
      Tobacco Fd-GOGAT 429 KIAQGAKPGEGGQLPGK KVSAYI A    RLRNSK 458  904 KVN K EIVKI Q R  914
        Pine Fd-GOGAT   1        PGEGGQLPGK KVSTYI A    RLRNSK  23  469 KVN K EIVKI Q R  479
```

Figure 10. Analysis of ferredoxin binding domains on glutamate synthase. Amino acid sequences of seven different GOGAT proteins were compared with six different ferredoxin-dependent proteins. Bold residues indicate electrostatic interaction with acidic residues on ferredoxin. An asterisk indicates identical residues or conserved substitutions of the following groups of amino acids (A, G), (V, I, L, M), (E, D), (N, Q), (W, F, Y)), (S, T) and (K, R).

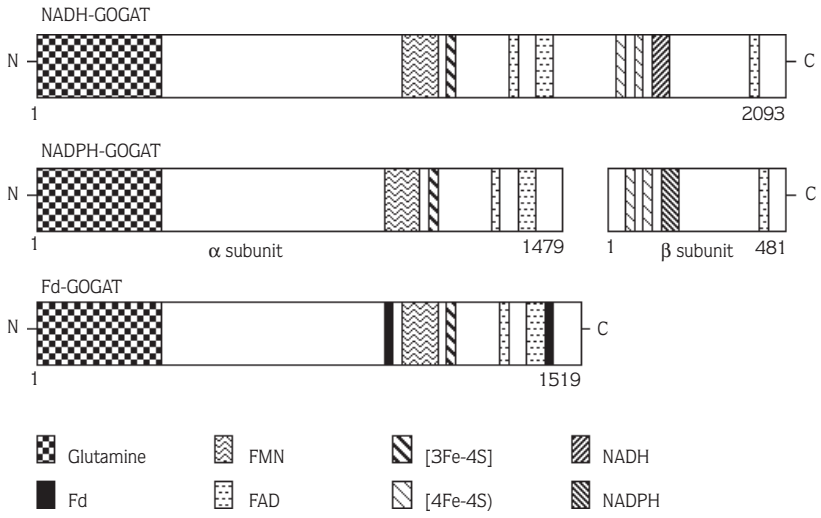

Figure 11. Identification of functional binding domains on NADH-, NADPH- and Fd-GOGAT proteins.

Conclusion

In this review, the published amino acid sequences of sixteen different NADH-,NADPH- and Fd-GOGAT proteins were analyzed to identify functional binding domains of glutamine, FMN, NAD(P)H, FAD, $[3Fe\text{-}4S]^{1+,0}$ and $[4Fe\text{-}4S]^{2+,1+}$ clusters and ferredoxin on this enzyme. It was determined that Fd-GOGAT has one domain each for glutamine, FMN and $[3Fe\text{-}4S]^{1+,0}$ cluster and two domains each for FAD and ferredoxin; NADPH-GOGAT α subunit has the same domains as Fd-GOGAT except for ferredoxin domains, and the β subunit has one domain each for NADPH and FAD and two domains for two $[4Fe\text{-}4S]^{2+,1+}$ clusters; NADH-GOGAT has the same domains as NADPH-GOGAT (Fig. 11). In the light of this analysis, it is therefore likely that Fd-, NADH- and NADPH-GOGATs share a common structure with regard to the catalytic and cofactor-binding domains but differ in terms of the chosen electron donor.

In two recent studies (55,56), kinetics analyses of bacterial NADPH-GOGATs performed using recombinant DNA techniques suggested that NADPH, NADH and Fd initially reduce FAD. Electron equivalents are then transferred through [Fe-S] centers to FMN. This flavin reduces the amino acid of glutamine and 2-oxoglutarate. Finally, 2 glutamates are produced. Since studies on GOGAT proteins are continuing with respect to how the reaction mechanism occurs and which amino acids are important during the reaction, this analysis of binding domains of substrate and cofactors will be helpful for further studies on GOGAT proteins.

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