Review

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Role of Chinese hamster ovary central carbon metabolism in controlling the quality of secreted biotherapeutic proteins

Recombinant proteins produced via Chinese hamster ovary (CHO) cell cultures constitute the majority of biotherapeutics on the market. The ability of these cultures to consistently perform human-like post-translational modifications, mainly glycosylation, is paramount to product quality. Although much research has focused on understanding enzymatic function and regulation in the glycosylation pathways of CHO cells, central carbon metabolism also plays an important role in determining protein quality by supplying nucleotide sugar precursors and generating inhibitory byproducts that affect protein glycosylation. Central carbon metabolism also generates reductant molecules that can promote disulfide bond cleavage during harvest, influencing the quality of the product. Through understanding CHO cell central carbon metabolism, host cell engineering strategies and process modifications have been developed to improve protein quality. This review highlights these advances and presents opportunities for future research toward understanding the role of central metabolism in controlling the quality of biotherapeutic proteins.

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The production of novel biotherapeutics using recombinant DNA technology has revolutionized modern medicine by treating a myriad of human diseases. These human proteins are typically produced in genetically modified eukaryotic host cells capable of complex post-translational modifications that are intrinsically impossible for simpler prokaryotic hosts to perform [1–3]. Mammalian cell lines, such as Chinese hamster ovary (CHO) cells, human embryonic kidney cells, baby hamster kidney cells and mouse myeloma cells (NS0), are typical choices for eukaryotic hosts because they are properly equipped to process complex biotherapeutic proteins [3–5]. Large-scale CHO suspension cultures have become the preferred production platform and are now used to produce approximately 60–70% of recombinant biotherapeutics on the market [3,4,6,7]. However, the higher titers now achieved in industrial-scale production to meet market demands often come at the price of compromised protein quality in aspects such as glycosylation [8].

The glycosylation of therapeutic proteins plays critical roles in the folding, immunogenicity, protease sensitivity [9], efficacy, *in vivo* half-life [4], solubility and thermal stability [10] of recombinant proteins. For these reasons, the extent and quality of glycosylation is an area of intense study in academia and industry [5,11,12]. Glycosylation is the process of adding oligosaccharides (i.e., glycans) to a polypeptide chain, as side chains covalently bonded to either asparagine or serine/threonine residues, termed *N*-linked or *O*-linked glycosylation, respectively. N-linked is the most common type of glycosylation in biotherapeutic proteins [13] as well as the most well studied due to its overwhelming impact on protein quality [4,5,14–18]. Due to the aforementioned implications of glycosylation, a thorough understanding of the metabolic

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Key Terms

Protein quality: Refers to the functionality of the protein in a pharmaceutical product. The extent, type and consistency of glycosylation are used synonymously with quality here, as it is one of the most commonly studied properties affecting protein quality.

Glycosylation: Post-translational modification undergone by proteins in which sugar chains (glycans) are added to the polypeptide chains of the proteins. Without proper glycosylation, the protein will not function correctly.

Disulfide bonds: Covalent bonds between two cysteine residues that maintain the 3D structure of a protein. Intrachain disulfide bonds link cysteine residues of the same peptide chain whereas interchain disulfide bonds link separate peptide chains together (e.g., the light and heavy chains of an antibody).

Galactosylation: Step preceding sialylation. The addition of a galactose molecule to the terminal site of the oligosaccharides. The terminal galactose provides the binding site for the terminal sialic acid added during sialylation.

Sialylation: Final step in glycosylation where sialic acid groups are added onto the glycans in the terminal position. This step is crucial for the functionality and half-life of biotherapeutic proteins and their acceptance by patients.

phenotypes that affect glycosylation is crucial to the biopharmaceutical industry.

In addition to the direct influence that expression level and activity of glycosylation enzymes have on protein quality, central carbon metabolism can also play an important role in controlling the quality of biotherapeutic proteins [19]. For example, the availability of nucleotide sugar building blocks is controlled by central metabolism. Culture supplements and metabolic engineering strategies have been successfully utilized to enhance the supply of these glycan precursors and remove metabolic bottlenecks [10,20–27]. Furthermore, ammonia production released during glutamine catabolism by CHO cell cultures is detrimental to protein quality [19,23,28–32]. However, the elimination of glutamine altogether can also negatively impact glycosylation [28]. Cellular metabolism also affects the downstream processing of biotherapeutic proteins. Reductive complexes released during product harvesting can disrupt the disulfide (DS) bonds that hold polypeptide chains together, thus destroying their functional 3D structure. Here again, host cell engineering strategies have successfully minimized DS bond reduction based on knowledge of the complex metabolic processes involved [33–38].

In this review, we will address the main pathways and mechanisms by which central carbon metabolism has been shown to influence product quality in CHO cell bioprocesses. In addition, we will highlight strategies that have been used to manipulate these pathways through metabolic engineering of host cells or process modifications that enhance the metabolic capabilities of CHO cultures, as summarized in Table I.

Metabolic supply of glycosylation precursors

Glycosylation, as diagrammed in Figure 1 occurs in the endoplasmic reticulum (ER) and Golgi apparatus as a sequence of enzymatic reactions necessary for biotherapeutic protein functionality [10,17]. Generally, in glycosylation the monosaccharides are combined with a nucleotide in order to form an activated nucleotide sugar molecule capable of binding to the polypeptide chain. These nucleotide sugars serve as the main precursors for the glycosylation reactions, as detailed in Figure 1. Activation of these monosaccharides to form nucleotide sugars occurs predominantly in the cytosol [10]. This compartmentalization makes the use of sugar-specific transporters necessary to facilitate the passage of the activated substrates into the Golgi apparatus from the cytosol [10]. All the monosaccharides required for glycosylation are either activated by a kinase or produced from a previously synthesized and activated nucleotide sugar. Therefore, the availability of the nucleotide sugar precursors and the sugar transporters are vital in regulating the extent and consistency of glycosylation of the protein product [10,29]. The following diverse methods for increasing precursor pool size and availability reflect the complexity of the metabolic pathways involved.

» Precursor supplementation

Precursor feeding studies have examined the role that nucleotide sugar availability has on the final steps of glycosylation, such as galactosylation and sialylation occurring in the Golgi apparatus [39]. UDP-*N*-acetylglucosamine (UDP-GlcNAc) is a common target for study because it is a precursor of the activated sugar donor required for attachment of sialic acid groups, cytidine 5′-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), as shown in Figure 1 [5,19,23]. Low levels of UDP-GlcNAc have been linked to low levels of terminal sialylation, and strategies for increasing the intracellular UDP-GlcNAc pool size have been implemented with varied success in CHO cell cultures.

One such strategy involves the supplementation of glucosamine (GlcN) or GlcN in combination with uridine (Urd) [21]. GlcN is a precursor for UDP-GlcNAc synthesis, which is the substrate for GlcNAc transferases that transport UDP-GlcNAc from the cytosol into the Golgi apparatus to be used in glycosylation [5]. When GlcN alone was supplemented to CHO cells, concentrations up to 5 mM caused no significant effect on cell

Table 1. Summary of metabolic treatments and effects on protein quality in recombinant Chinese hamster ovary cell cultures.

Treatment	Effect	Ref.
2.5 mM GlcN	Eight-times increase in UDP-GNAc pool size	[40]
10 mM GlcN	Approximately eight times increase in UDP-GNAc pool size Decrease in sialylation Decrease in antennarity Increase in protein heterogeneity (molecular weight and pI ranges)	[40]
10 mM GlcN and 2 mM Urd	Increased GlcNAc pool size Decreased growth rate 45% decrease in protein yield Decreased specific GUR Approximately 5–8% decrease in terminal sialylation Increase in antennarity	[21,31]
15 mM NH_4Cl and 0.5 mM Ado	Low, constant UDP-GNAc pool Tendency toward more homogeneous product Decrease in sialylation	[31]
20 mM ManNAc	Increased CMP-sialic acid pool size: 12-fold to 30-fold Increase in protein sialylation	[20,21]
20 mM ManNAc and 10 mM Cyt	Increased CMP-sialic acid pool size Approximately 8% increase in terminal sialylation 26–52% increase in specific productivity	[39]
CMP-SAT overexpression	4–16% increase in terminal sialylation	[10]
α(2,3)-ST overexpression	Increase in sialylation	[27]
α(2,3)-ST and CMP-SAS overexpression	No further increase in sialylation from α(2,3)-ST overexpression alone Increase in intracellular sialic acid concentration	[27]
α(2,3)-ST and CMP-SAS + CMP-SAT overexpression	Increase in sialylation compared with α(2,3)-ST + CMP-SAS overexpression 1.5-fold increase in sialic acid concentration compared with control with no metabolic manipulations	[27]
Rat GNE/MNK + α(2,3)-ST and CMP-SAT overexpression	>tenfold increase in sialic acid concentration 43% increase in product protein sialic acid content	[26]
30 mM NH_4Cl	Approximately eight-times increase in UDP-GNAc pool size Decrease in sialylation Decrease in antennarity (More pronounced effects than the 10 mM GlcN cultures)	[40]
10 mM NH_4Cl	Reduced growth rate Initially decreased *CMP-SAT* gene expression before leveling off at 49 h Decreased α(2,3)-ST expression throughout culture	[30]
TXN1 KD	Inhibition of DS bond reduction event by 75% at 24 h Decrease in efficacy at 15E6 cells/ml	[36]
20 mM EDTA	Inhibited DS bond reduction event for 2 days in 75% total cell lysis conditions	[35]
EDTA and G6P	No effect on DS bond reduction	[34]

α(2,3)-ST: α(2,3)-sialyltransferase; Ado: Adenosine; CMP: Cytidine monophosphate; CMP-SAS: Cytidine monophosphate sialic acid synthase; CMP-SAT: Cytidine monophosphate sialic acid transporter; Cyt: Cytidine; DS: Disulfide; G6P: Glucose-6-phosphate; GlcN: Glucosamine; GlcNAc; *N*-acetylglucosamine; GNE/MNK: UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase; GUR: Glucose uptake rate; KD: Knockdown; ManNAc: *N*-acetylmannosamine; TXN1: Thioredoxin 1; UDP-GNAc: UDP-*N*-acetylglucosamine; Urd: Uridine.

growth, but significant growth inhibition was observed at GlcN concentrations of 10 mM and above [40]. The reduced growth rate has been linked to a decrease in specific glucose uptake rate (GUR) [21], likely due to GlcN competitively inhibiting glucose transport into the cell [31,40]. GlcN supplementation has also been observed to yield greater protein heterogeneity and an eightfold increase in the combined UDP-GlcNAc and UDP-*N*-acetylgalactosamine (UDP-GalNAc) pool (together denoted as UDP-GNAc) [40]. However, as the concentration of supplemented GlcN increased, the productivity of the cells decreased [40].

It has also been hypothesized that the synthesis of UDP-GlcNAc from GlcN could trap UTP in the

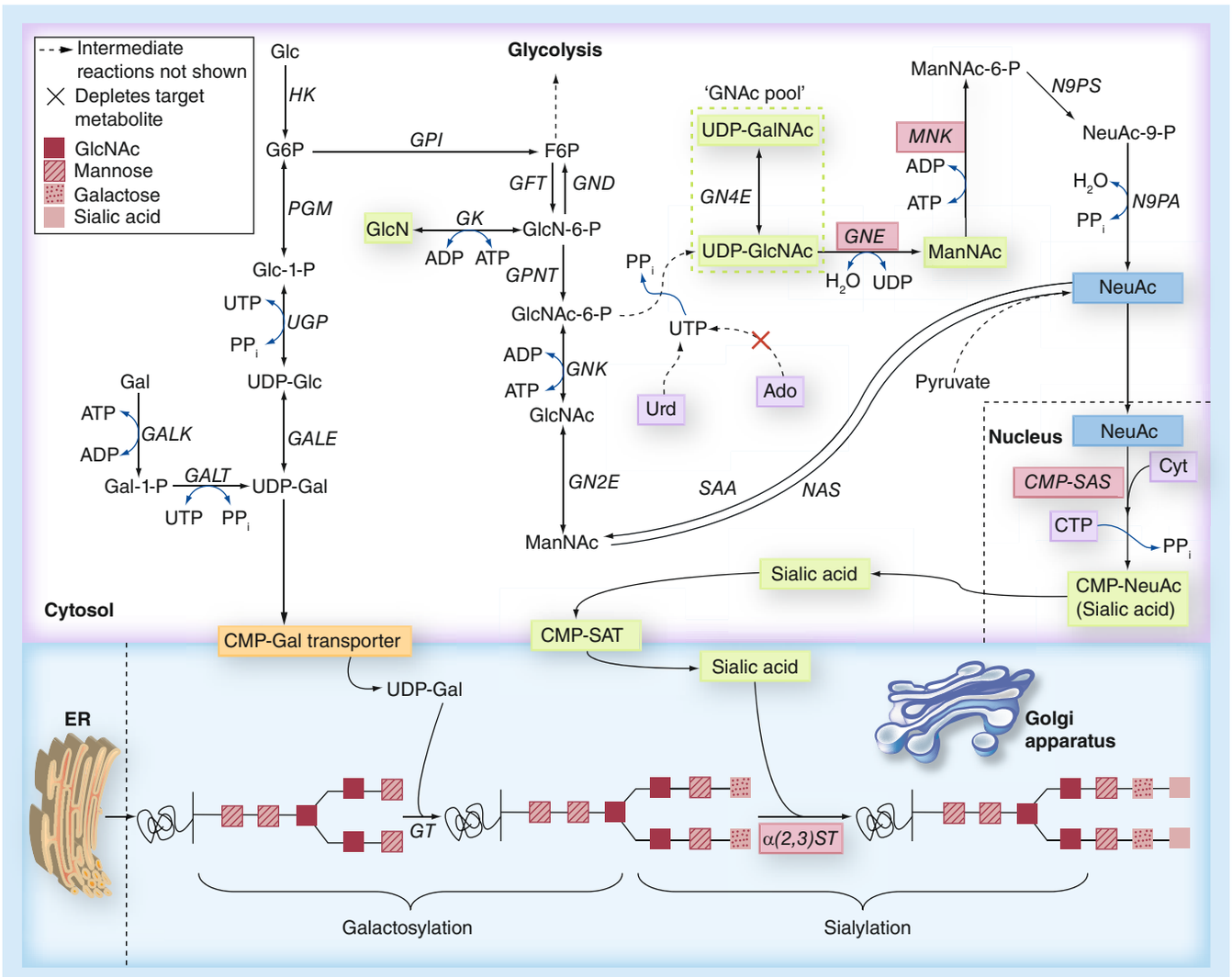

Figure 1. Sialic acid biosynthesis with a simplified view of the final steps in glycosylation as discussed in this review. CMP-SAT facilitates the transport of the sialic acids synthesized in the cytosol to the Golgi apparatus where sialylation occurs. Metabolites in shaded boxes indicate precursors discussed in this review; italicized species indicate enzymes; broken arrows indicate that intermediate reactions between substrate and product are not shown; blue arrows and species indicate side reactions that donate or accept phosphate groups for use in the main metabolic pathways shown.

α(2,3)-ST: α(2,3)-sialyltransferase; Ado: Adenosine; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CMP: Cytidine monophosphate; CTP: Cytidine triphosphate; Cyt: Cytidine; ER: Endoplasmic reticulum; F6P: Fructose-6-phosphate; G6P: Glucose-6-phosphate; Gal: Galactose; GALE: Uridine diphosphate-galactose 4-epimerase; GALK: Galactokinase; GalNAc: *N*-acetylgalactosamine; GALT: Galactose-1-phosphate uridylyltransferase; GFT: Glutamine–fructose-6-phosphate transaminase; GK: Glucosamine kinase; Glc: Glucose; GlcN: Glucosamine; GlcNAc: *N*-acetylglucosamine; GN2E: *N*-acetylglucosamine 2-epimerase; GN4E: *N*-acetylglucosamine 4-epimerase; GNAc: *N*-acetylglucosamine and UDP-*N*-acetylgalactosamine; GND: Glucosamine-6-phosphate deaminase; GNE: UDP-*N*-acetylglucosamine 2-epimerase; GNK: *N*-acetylglucosamine kinase; GPI: Glucose-6-phosphate isomerase; GPNT: Glucosamine-phosphate *N*-acetyltransferase; GT: Galactosyltransferase; HK: Hexokinase; ManNAc: *N*-acetylmannosamine; MNK: *N*-acetylmannosamine kinase; N9PA: *N*-acetylneuraminic acid-9-phosphate-phosphatase; N9PS: *N*-acetylneuraminic acid-9-phosphate-synthase; NAS: *N*-acetylneuraminic acid synthase; NeuAC: *N*-acetylneuraminic acid; PGM: Phosphoglucomutase; PP_i: Pyrophosphate; SAA: Serum amyloid A; SAS: Sialic acid synthase; SAT: Sialic acid transporter; UDP: Uridine diphosphate; UGP: Uridine diphosphate-glucose pyrophosphorylase; UTP: Uridine triphosphate; Urd: Uridine.

UDP-GlcNAc pool, which would result in depletion of UTP in the cell [21]. Because of this, Urd is sometimes added alongside GlcN because it mitigates the depletion of intracellular UTP, which is necessary for the activation of *N*-acetylglucosamine-1-phosphate to the glycosylation precursor UDP-GlcNAc [21,31]. While GlcN and Urd supplementation did result in an increased UDP-GlcNAc pool, it also resulted in a reduced growth rate [21,31,39], a 45% decrease in protein yield, and a slight (5–8%) decrease in terminal

sialylation [21]. Direct measurements of intracellular nucleotide phosphate and nucleotide sugar concentrations would therefore be highly informative for debottle necking this process, since Urd was intended to mitigate the UTP depletion yet no improvement in sialylation was observed. When UTP was intentionally depleted by adenosine addition, the UDP-GNAc pool was kept low and constant [31]. Under these conditions, the sialylation was still decreased even though additional UDP-GNAc was not formed through UTP action.

Another method of increasing precursor supply is through supplementation of 20 mM *N*-acetylmannosamine (ManNAc), a direct precursor for sialic acid [10,20,21]. ManNAc supplementation has increased the intracellular pool of CMP-sialic acid (CMP-NeuAc) by 12- to 30-fold [20,21] in protein-producing CHO cell cultures. This enhanced sialic acid pool size led to an increase in protein sialylation in both cases [21,22], indicating that sialic acid pool size can be a limiting factor in the sialylation of glycoproteins. The ManNAc spikes did not have an effect on cell growth, viability, or antennarity in either of the aforementioned studies [20,21]. As can be seen in **Figure 1**, ManNAc must be converted to NeuAc and subsequently activated by cytidine triphosphate (CTP) to form the nucleotide sugar CMP-NeuAc, which is the major sialic acid donor in glycoprotein synthesis. Because of this activation step, any depletion of CTP would limit the amount of sialic acid formed. Wong *et al.* fed CHO cells 20 mM ManNAc in conjunction with 10 mM cytidine (Cyt) to mitigate the depletion of CTP [39]. Its addition led to a larger intracellular pool of sialic acid and an approximately 8% increase in sialylation of the product protein [39]. This ManNAc and Cyt feed also led to 26–52% higher specific productivities than the Cyt-free control cultures [39].

Key Term

Antennarity: Term that encompasses the branching of glycans (sugarside chains) attached to the polypeptide chain during glycosylation. The number of antennae on a given glycoprotein is sometimes used to quantify the antennarity. Also referred to as complexity.

» Metabolic engineering for enhanced precursor supply

As was previously mentioned and illustrated in **Figure 1**, sialic acids are synthesized in the cytosol of the cells. They must be transported by nucleotide sugar transporters into the Golgi apparatus to be properly incorporated into the glycosylated product protein [10,24]. The endogenous CMP-sialic acid transporter (CMP-SAT) in CHO cells has been shown to facilitate the transport of sialic acid into the Golgi apparatus [10,26,27]. Overexpression of CMP-SAT in one prior study increased the pool of sialic acid available for sialylation and led to a 4–16% increase in sialylation of the product protein [10]. Some clones overexpressing CMP-SAT also produced a lower proportion of non-sialylated glycans compared with the parental lines with no overexpression of CMP-SAT in that same study [10].

The results of CMP-SAT overexpression confirmed that sialic acid transport into the Golgi apparatus could be a limiting factor in sialylation. However, once inside the Golgi apparatus, other enzymes are responsible for catalyzing the actual sialylation reactions. These sialylation enzymes provide other potential bottlenecks in the glycosylation pathway. Particularly, after CMP-SAT transports the sialic acid into the Golgi apparatus, it is attached to a previously galactosylated protein by α(2,3)-sialyltransferase (α(2,3)-ST) [27]. A gene expression study shows that α(2,3)-ST is a naturally occurring bottleneck [30], and increases in sialylation have been reported with α(2,3)-ST overexpression [10,27]. When combined with overexpression of CMP-sialic acid synthase (CMP-SAS), an enzyme that functions in sialic acid synthesis, no increase in sialylation was observed over α(2,3)-ST overexpression [27]. However, an increase in intracellular sialic acid pool size was observed, confirming that CMP-SAS stimulates sialic acid production [27]. This result, combined with other previous findings [27,30,41] and substantiated by the previously mentioned gene-expression study [30], leads to the conclusion that a bottleneck exists at the CMP-SAT step responsible for transporting sialic acid into the Golgi apparatus. Subsequent co-overexpression of CMP-SAT along with α(2,3)-ST and CMP-SAS led to no significant growth or productivity effects with a 1.5-fold increase in intracellular sialic acid pool size and increased sialylation relative to the control [27].

It was hypothesized that the aforementioned co-overexpression of CMP-SAT along with α(2,3)-ST and CMP-SAS did not provide the desired increase in sialylation because of feedback inhibition of the enzyme responsible for the initial two steps of sialic acid synthesis, UDP-GlcNAc 2-epimerase (GNE)/ManNAc kinase (MNK) [26,27]. Because it is feedback inhibited by sialic acid, GNE/MNK is incapable of synthesizing substantially increased quantities of sialic acid required to boost sialylation. Mutated rat GNE/MNK genes that lacked feedback inhibition were co-overexpressed with α(2,3)-ST and CMP-SAT in CHO cells to eliminate the sialylation bottleneck [26]. This triple overexpression led to more than a tenfold increase in intracellular sialic acid pools and a 43% higher sialic acid content in the product protein [26].

This strategy is very promising for use in the production of highly sialylated biotherapeutic proteins from CHO cells.

Inhibition of glycosylation via metabolic byproducts

Metabolic byproducts naturally produced and accumulated by CHO cell cultures affect the quality of the product protein by changing the culture environment and ultimately altering metabolic pathways that control post-translational modifications. The two most important metabolic byproducts of CHO cell cultures are ammonia and lactate. While ammonia directly impacts protein quality through inhibition of glycosylation enzymes, lactate has shown no direct inhibition of protein quality [42]. However, to combat high lactate concentrations that negatively impact cell growth and protein productivity, glucose supply to CHO cultures is often limited. This can have the unwanted side effect of restricting the availability of glycosylation precursors needed to maintain protein quality.

» Effects of ammonia on glycosylation quality

Ammonia is a naturally occurring byproduct of amino acid metabolism and of the chemical breakdown of glutamine in the media [30,32,43]. High ammonia concentrations can increase the antennarity of product proteins when coupled with an increase in UDP-GNac pool size [31,40]. Furthermore, ammonia interferes with terminal sialylation – the final step in glycosylation that, when properly completed, prevents rejection of the protein by human recipients [5,9,23,29,31,42]. Ammonia concentrations of 8–10 mM [44,45] to as high as 20 mM [16] are regularly produced in CHO suspension cultures. While 4–8 mM ammonia is inhibitory to growth [46,47], a concentration as low as 2 mM raises the pH of the Golgi apparatus enough to prevent optimal activity of the sialyltransferase enzymes, such as α(2,3)-ST [5,43]. The effects of ammonia concentration are even more pronounced at higher concentrations – at 20mM, both antennarity and sialylation are impaired [9,28].

One previous study suggested that ammonium concentrations affect the expression of glycosylation genes located inside the Golgi apparatus [30]. Decreased expression levels of CMP-SAT indicated that ammonium impairs the final transport of sialic acid from the cytosol into the Golgi apparatus, where it is ultimately incorporated into the product protein [30,48]. Furthermore, sialidase, the enzyme responsible for desialylating secreted proteins, was found to be insensitive to elevated ammonium levels, indicating that increased desialylation was not the cause of the observed decrease in sialylation under high ammonium conditions [30]. Instead, this result indicates that bottlenecks within the sialylation pathway, particularly at CMP-SAT and α(2,3)-ST, cause a decrease in sialylated product in the presence of elevated ammonium [30].

» Growth in the absence of glutamine

Glutamine is most often cited as the most abundantly consumed amino acid in immortalized cell cultures [49]. In a dihydrofolate reductase (DHFR)-deficient CHO clone, glutamine made up 25% of the total carbon consumed during the exponential growth phase [50]. Unsurprisingly, it is added in substantial concentrations to various growth media. The consumption of glutamine greatly exceeds biosynthetic demands [51,52], suggesting it plays an important catabolic role within the cell. Glutamine serves as a complementary energy source to glucose that can feed directly into the tricarboxylic acid cycle, enhancing growth and viability of CHO cultures [23,28]. Due to the substantial glutamine that is consumed, excess ammonia often accumulates in culture, which can negatively affect the glycosylation quality of product proteins. Furthermore, non-enzymatic glutamine degradation makes a nontrivial contribution to ammonia production, since nearly 25% of the extracellular glutamine can spontaneously degrade in only 3 days of culture [50]. As a result, several studies have been performed under low or nonexistent glutamine concentrations in order to mitigate the negative effects of ammonia accumulation on protein glycosylation [19,23,28].

Studies have found that CHO cultures acclimated to glutamine-depleted conditions experience lower ammonia and lactate accumulation and a slower decrease in pH [19,23,28]. After adapting cell cultures to glutamine-free media, cell metabolism is expected to shift toward glycolysis leading to higher glucose consumption [28]. This predicted increase in glucose consumption stems from the joint regulation of glycolysis and glutaminolysis (glutamine catabolism) to provide energy for the cell [53]. However, studies have shown no significant growth rate discrepancies or glucose uptake rate variations among parental cell lines and those acclimated to glutamine-deficient environments [19,23,28]. In fact, one study observed no increase in glucose consumption nor a more efficient use of glucose, with the ratio of lactate produced to glucose consumed remaining constant [28]. In this study, cells compensated for the lack of glutamine by increasing their consumption of glutamate, aspartate and asparagine [28]. Although specific protein productivity was not significantly different in the glutamine-deficient cultures, lower ammonia production was associated with a prolonged growth phase and increased viable cell density of these cultures [28].

Another study involving a different biotherapeutic protein displayed a decrease in glycolytic flux as well as a decrease in the intracellular concentration of UDP-GlcNAc as a result of glutamine-deficient growth conditions [19]. As discussed in the previous section, any decrease in UDP-GlcNAc concentration can adversely affect the glycosylation and sialylation of proteins. Therefore, improvements in protein quality that might be accrued from reducing ammonia in glutamine-depleted cultures could be offset by the lack of available substrate for glutamine-dependent glycosylation pathways, such as UDP-GlcNAc synthesis. Understanding this tradeoff between cell growth, productivity and product quality should be an important future goal of media and process optimization, especially when manipulating the glutamine supply to the culture.

» Glutamine synthetase & its ability to reduce ammonia accumulation

As an alternative to gradually adapting CHO cell cultures for growth in glutamine-free media, glutamine synthetase (GS) overexpression has become a popular approach for conferring glutamine-independent growth, and is considered one of the great successes of mammalian host cell engineering [54]. Specifically, the GS enzyme catalyzes direct glutamine synthesis from glutamate, ammonia and ATP in the presence of Mg^{2+}. CHO cells are capable of endogenous GS expression, as confirmed by the CHO-K1 genome [55]. In most cases, however, expression and activity of GS are not sufficient to maintain maximal growth [23]. Therefore, recombinant GS overexpression enhances the ability of CHO cells to synthesize glutamine from glutamate in the absence of an extracellular glutamine supply. This has obvious potential to reduce glutamine catabolism, and therefore ammonia accumulation, without diminishing the overall growth rate. Furthermore, GS activity consumes free ammonia from the cellular environment, and with glutamine removed from the culture medium, non-enzymatic ammonia production is substantially reduced [56]. In some cases, the complete elimination of ammonia production has been reported [49,57]. However, despite the success of GS in reducing ammonia production, the effect of GS expression on protein glycosylation has not been systematically studied to our knowledge.

» Minimizing lactate by limiting glucose availability

Lactate is widely known to inhibit CHO cell growth [51,52], and minimizing lactate accumulation has been a primary focus of cell culture engineering for many years [7,44,58–61]. One of the more popular strategies used to limit lactate production in mammalian cell cultures is to restrict glucose availability, as discussed in two recent reviews [6,40]. Another similar strategy is to feed an alternative sugar that is metabolized more slowly than glucose: galactose, mannose or fructose, for example [59]. While both approaches are effective in limiting lactate accumulation, they can negatively impact glycosylation quality. For example, substituting another sugar for glucose has been shown to limit the combined pool size of UDP-GNAc [62]. Both of these activated sugars are essential precursors that feed into glycosylation pathways and are interconverted freely, as shown in **Figure 1**. In addition, feeding galactose, even in conjunction with glucose, has been shown to enhance desialylation due to increased sialidase activity observed under galactose-fed conditions [63]. When glucose availability is limited, oligosaccharides that typically attach at asparagine residues have been found to be either truncated or missing [62,64,65].

Considering that all of the aforementioned studies were performed in various CHO cell lines producing diverse products, the implications of these findings are substantial. The practice of limiting glucose availability to reduce lactate accumulation could have unintended consequences on glycosylation quality in a wide range of instances. Sensitivity to glycosylation quality is one of the reasons that simpler fed-batch systems are exchanged for perfusion reactors in the production of some proteins. Perfusion reactors allow glucose concentration to be constantly maintained over long periods of time and thereby reduce product variability. However, this comes at the expense of increased downstream processing costs and process complexity [66,67].

Many metabolic engineering strategies have been applied to decrease the lactate accumulation in CHO cell cultures [49]. However, to our knowledge, no studies have specifically examined the effects of these manipulations to alter product quality in conjunction with their ability to reduce lactate production.

» DS bond reduction

Another way that central carbon metabolism impacts protein quality is through its effect on DS bond reduction. Inter-chain DS bonds are largely responsible for linking the light and heavy chains of an antibody together to form its functional 3D structure [33–36]. These bonds are formed in the oxidizing environment of the ER and offer structural stability to many secreted proteins. Upon harvest of the proteins, there can be a significant DS bond reduction event caused by the lysing of cells during the mechanical harvesting procedure [34–36]. The reduction of these DS bonds can cause the proteins to dissociate and lose functionality. The reduction event is not specific

to one particular product protein and occurs during both manufacturing-scale and laboratory-scale production runs [35,36].

DS bonds are formed from two cysteine residues via oxidation of their thiol side groups. Re-oxidizing the reduced proteins downstream is an undesirable process because it is difficult to control and may cause unwanted oxidation of other amino acid residues, further compromising protein quality [35]. Because of this, the metabolic pathways responsible for DS bond reduction have been studied in order to metabolically engineer CHO cells that lack this tendency.

Since DS bond reduction is not ubiquitous across all CHO cell lines and/or culture conditions, studies have been performed to identify metabolic phenotypes that are unique to cultures that consistently reproduce this reduction event [34–36]. These studies have shown that an active thioredoxin (Trx) system – consisting of Trx, thioredoxin reductase (TrxR) and NADPH – is present in the reducing cell culture medium as shown in Figure 2 [34,36]. The Trx system is an abundant antioxidant system that is important in maintaining the redox state of the cell as well as maintaining the general reduction of other intracellular DS proteins. Unfortunately, Trx remains active in the cell culture medium after cell lysis [34,37]. The NADPH required for Trx system activity is provided during the conversion of NADP+ and glucose-6-phosphate (G6P) via G6P dehydrogenase (G6PD) to NADPH and 6-phosphoglucono-δ-lactone via a reaction that normally functions within the pentose phosphate pathway of intact cells [34]. G6P is largely produced from glucose and ATP via hexokinase (HK) activity in the first step of glycolysis. When cells lyse, these enzymes are released into the cell culture medium along with their substrates and continue catalyzing reactions, fueling the production of NADPH that is used by the Trx system. Based on these observations, inhibition or elimination of the Trx system, including central metabolic pathways that supply NADPH, could result in improved stability of DS bonds during and after harvest.

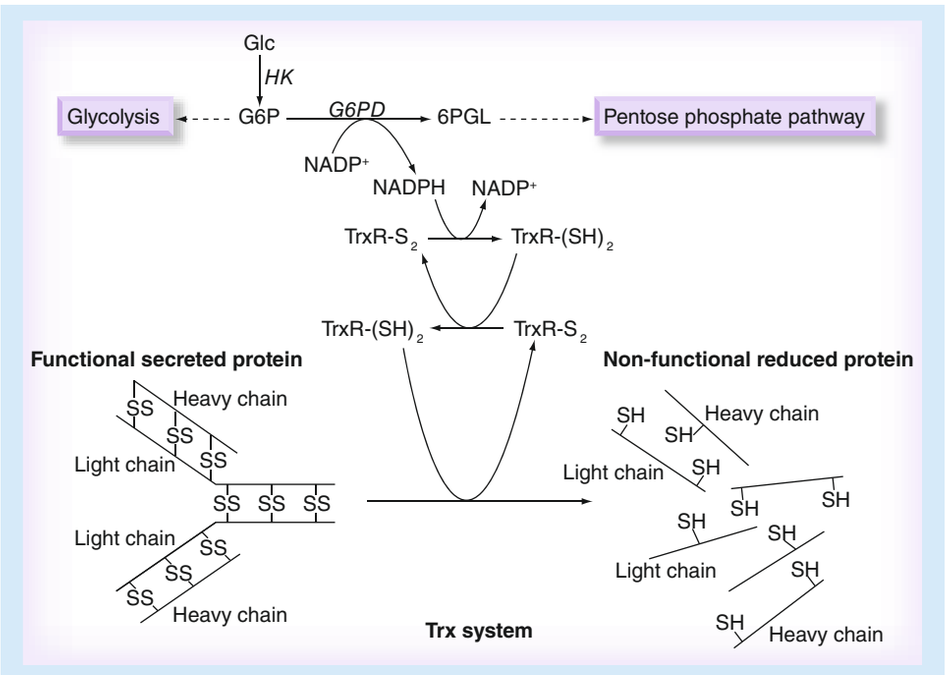

Figure 2. The thioredoxin system is responsible for the disulfide bond reduction mechanism that can occur in harvested Chinese hamster ovary cell culture medium. The NADPH that initiates the Trx system is produced by the first steps of glycolysis and the pentose phosphate pathway. TrxR reduces Trx using NADPH as its cofactor. The reduced Trx then acts as the terminal enzyme responsible for catalyzing the disulfide bond reduction event.
6PGL: 6-phosphogluconolactone; G6P: Glucose-6-phosphate; G6PD: Glucose-6-phosphate dehydrogenase; Glc: Glucose; HK: Hexokinase; Trx: Thioredoxin; TrxR: Thioredoxin reductase.

» Metabolic engineering to prevent DS bond reduction

The oxidoreductase enzyme thioredoxin 1 (TXN1) is the terminal enzyme of the Trx system that is responsible for the reduction event in CHO cell cultures as shown in [36]. With the knockdown (KD) of TXN1 expression via lentiviral transduction of short hairpin RNA, the overall growth of the cells was not negatively affected, and the highest level of TXN1 KD efficiency (95.3%) inhibited DS bond reduction by 75% after a 24 h incubation [36]. However, TXN1 KD lost efficacy at cell densities above 15 × 10^6 cells/ml [36]. The KD of TXN1 was not reported past a 24 h incubation time, but data show that at 24 h it was already losing its effect [36]. This implies that a prompt removal of the product protein from the harvest cell culture medium is vital in maintaining the product's structural integrity and, therefore, functionality.

» Chemical inhibition of DS bond reduction

Along with metabolic engineering strategies, chemical inhibitors and chemical oxidizing agents have been applied to prevent the DS bond reduction event. The metal chelator and protease inhibitor EDTA effectively inhibits the reduction event when added to cell culture medium [34]. EDTA

added to both the pre-harvest culture medium and the harvested medium at a concentration of 20 mM effectively inhibited DS bond reduction for up to 2 days despite approximately 75% cell lysis [34]. In contrast, the product was completely reduced after only 1 day in the absence of EDTA addition [35]. Interestingly, combinatorial addition of EDTA and G6P did not eliminate the reduction event, suggesting that EDTA acts primarily by inhibiting G6P production by HK [34]. This, in turn, cripples the Trx system by eliminating NADPH production in the harvested medium [34].

Future perspective

There is now a proliferation of success stories in the mammalian biotechnology industry where recombinant antibody titers have been improved up to and beyond the 5 g/l level [67]. Further improvements in product titers are still achievable, which would provide additional manufacturing capacity and cost savings to what is now a US$100 billion global biotherapeutics industry [6]. However, a broader view of cell culture engineering that encompasses improved product quality and consistency, in addition to yield improvements, has now firmly taken root. The efficient production of protein therapeutics hinges upon the ability of CHO cells and other mammalian cell lines to produce ever-increasing amounts of product with consistent quality. This places a substantial burden on the metabolic pathways of the host cell to not only meet the bioenergetic demands of growth and protein production, but also to supply the building blocks for glycosylation and post-translational processing of proteins.

Although there has been much prior effort to understand and engineer glycosylation pathways to improve product quality, very few studies have directly examined how redirecting glucose and glutamine metabolism impacts the supply of nucleotide sugars needed for synthesis of complex glycans

Executive summary

Background

- Chinese hamster ovary (CHO) cells are the predominant host cell line for biotherapeutic protein production in the pharmaceutical industry.
- The quality of biotherapeutic proteins is often dictated by the extent and pattern of glycosylation.
- Glycosylation can be affected by many factors, including central metabolic processes that control the production of nucleotide sugar precursors and the accumulation of inhibitory byproducts such as ammonia.
- Proteins can also be reduced in an NADPH- and thioredoxin-dependent manner upon harvest, rendering the proteins nonfunctional.

Availability of glycosylation precursor substrates

- Nucleotide sugar substrates determine the amount and rate of glycosylation occurring in the cells.
- Feeding nucleotide sugar precursors has proved successful in increasing intracellular pool sizes and increasing the amount of sialylation of recombinant proteins.
- Metabolic engineering techniques have also been implemented to aid in the transport of substrates into the endoplasmic reticulum and Golgi for proper glycosylation.

Inhibition of glycosylation via metabolic byproducts

- Ammonia concentrations as low as 2 mM can affect the sialylation and, therefore, the glycosylation of biotherapeutic proteins.
- To combat ammonia's effect on protein quality, cells have been cultured in media devoid of glutamine. While effective in limiting ammonia accumulation, growth is reduced and glycosylation quality decreases.
- Overexpression of GS eliminates the need to provide high glutamine concentrations in the media by providing catalytic activity to synthesize glutamine endogenously. This limits glutamine catabolism and resulting production of ammonia.
- Lactate is a second metabolic byproduct of CHO cell culture. Limiting the glucose supply to cultures is often used to diminish the production of lactate.
- Glucose deprivation has been directly linked to decreased levels of glycosylation and, therefore, diminished protein quality.

Disulfide bond reduction

- Disulfide (DS) bonds represent an important structural feature that holds proteins together, and any reduction of the DS bonds causes the proteins to break apart, rendering them useless.
- Upon mechanical harvesting of biotherapeutic proteins, there is an unavoidable amount of cell lysis that occurs. This releases the intracellular contents into the harvest medium and can cause reduction of DS bonds if an active thioredoxin system is present in the cells.
- Through metabolic engineering of CHO cells, thioredoxin system activity can be inhibited thus diminishing the likelihood of DS bond reduction.

[5,68], or other aspects of protein quality such as post-harvest reduction of DS bonds. It is likely that future metabolic engineering strategies will place equal emphasis on understanding the role of central metabolism in controlling both the quality as well as the shear amount of product that can be manufactured by a recombinant host. This will include the application of isotope tracers and ^{13}C metabolic flux analysis (MFA) to map the flow of carbon and nitrogen into biosynthetic precursors [6,50,69]. Indeed, two recent studies have already applied ^{13}C MFA to investigate the metabolic supply of GlcNAc and other nucleotide sugars required for protein glycosylation [11,19]. These, and other future studies, are expected to reveal novel process optimizations and metabolic engineering strategies that can enhance protein quality while simultaneously meeting the bioenergetic demands for high cell-specific productivity, cell growth and product titer.

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