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REVIEW ARTICLE

Review of Fructosyl Amino Acid Oxidase Engineering Research: A Glimpse into the Future of Hemoglobin A1c Biosensing

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Abstract

Glycated proteins, particularly glycated hemoglobin A1c, are important markers for assessing the effectiveness of diabetes treatment. Convenient and reproducible assay systems based on the enzyme fructosyl amino acid oxidase (FAOD) have become attractive alternatives to conventional detection methods. We review the available FAOD-based assays for measurement of glycated proteins as well as the recent advances and future direction of FAOD research. Future research is expected to lead to the next generation of convenient, simple, and economical sensors for glycated protein, ideally suited for point-of-care treatment and self-monitoring applications.

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Introduction

Hyperglycemia is responsible for most of the symptoms and long-term complications of diabetes. It is well recognized that adequate metabolic control of the blood glucose level in diabetes patients can delay and even prevent the onset of long-term complications.[1] Adequate glycemic control also resulted in perceived improvements in overall quality of life.[2] Early diagnosis and regular assessment of treatment effectiveness are therefore very important for the prevention of these serious complications.

Self-monitoring of blood glucose is an integral part of a structured self-management strategy for achieving target glycemic levels. However, assessing treatment effectiveness relies on a method that can determine the average blood glucose concentration over an extended period. Long-term hyperglycemia from poor glycemic control results in decreased concentrations of the nonmetabolizable sugar 1,5-anhydro-D-glucitol (1,5-AG). Enzyme assay kits are available for measuring 1,5-AG in blood,[3] providing an assessment of the overall glycemic control over the past few days to 2 weeks. Treatment assessment is also increasingly done by measuring glycated serum albumin, whose levels are proportional to the average blood glucose concentration of the preceding 1–2 weeks. However, by far the preferred method of

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Abbreviations: (1,5-AG) 1,5-anhydro-D-glucitol, (f-$^{\varepsilon}$Lys) fructosyl ε-lysine, (f-$^{\alpha}$Val) fructosyl valine, (f-$^{\alpha}$Val-His) fructosyl valyl histidine, (FAD) flavin adenine dinucleotide, (FAOD) fructosyl amino acid oxidase, (FPOX) fructosyl peptide oxidase, (A1C) glycated hemoglobin A1c, (HPLC) high-performance liquid chromatography, (mPMS) methoxy-5-methyl phenazinium methyl sulfate

Keywords: biosensing, fructosyl amino acid oxidase, glycated proteins, hemoglobin A1c, A1C, point-of-care treatment

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evaluating the treatment effectiveness is measuring the glycated hemoglobin A1c (A1C), which has been for some time the gold standard for assessing long-term control of glycemic levels in diabetes patients. The American Diabetes Association currently recommends maintaining an A1C level below 7% of total hemoglobin.[4]

Glycated hemoglobin A1c is a hemoglobin molecule in which the *N*-terminal valine residue of the β subunit has been modified by blood glucose (**Figure 1A**). This modification, called glycation to distinguish it from the enzymatic glycosylation of proteins, is a nonenzymatic reaction of glucose with free amino groups, proceeding through a Schiff base intermediate to produce a relatively stable product. Easily separated from the unglycated form due to its lower pI value, A1C was initially identified as the most abundant of minor negatively charged hemoglobin components that eluted before the main hemoglobin peak during cation exchange chromatography.[5] Even before it was determined to be modified with glucose, A1C was observed in significantly greater amounts in patients with diabetes.[6–8] Due to the erythrocyte's long lifetime and the slow continuous and essentially irreversible characteristics of the glycation process,[9] the relative amount of A1C reflects the average blood glucose concentration of the past 2–3 months.[10,11]

Clinical laboratories have been using a number of different A1C measurement systems, the major ones being ion-exchange high-performance liquid chromatography (HPLC), immunoassay, and boronate affinity chromatography. Immunological methods, which have recently become more popular, can be employed with clinical automated analyzers for measuring a large number of samples in a short time. However, this method is relatively costly, and its results are affected by the presence of hemoglobin variants. High-performance liquid chromatography methods, which are the most commonly used, offer high reproducibility and accuracy, and most are now unaffected by hemoglobin variants. However, HPLC methods involve expensive equipment, requiring specially trained staff and relatively long operating times.

Commercially available enzyme assay systems have offered an attractive alternative for conventional clinical tests for glycated proteins. These tests, based on the enzyme fructosyl amino acid oxidase (FAOD), are rapid and have been shown to be reproducible.[12–14] There is active research trying to improve the properties of this enzyme and developing novel FAOD-based detection systems. Fructosyl amino acid oxidase is expected to become a major component of glycated protein sensing and eventually be applied in simple, convenient, and economical detection systems for point-of-care treatment and self-monitoring applications. This article reviews past, current, and expected future research in the field of FAOD-based sensors for glycated protein.

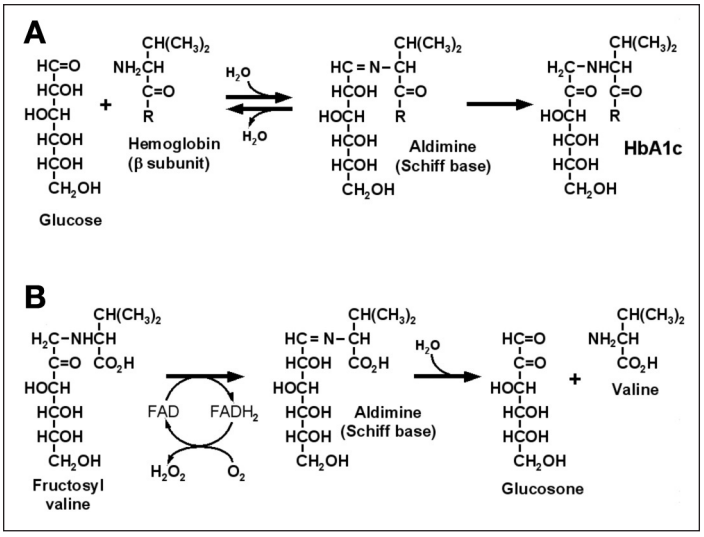

Figure 1. The chemical reactions behind the synthesis and FAOD-based sensing of A1C. **(A)** The nonenzymatic glycation of the *N*-terminal valine residue of the hemoglobin β subunit, resulting in A1C. **(B)** Fructosyl-amino-acid-oxidase-catalyzed oxidation of f-αVal that is released by the proteolytic digestion of A1C.

Enzyme Properties of Fructosyl Amino Acid Oxidase

Fructosyl amino acid oxidase catalyzes the oxidation of the C–N bond linking the C1 of the fructosyl moiety and the nitrogen of the amino group of fructosyl amino acids (**Figure 1B**). The reaction proceeds to an unstable Schiff base intermediate, which hydrolyzes to produce glucosone and an amino acid. The enzyme's reduced flavin adenine dinucleotide (FAD) cofactor is then reoxidized by molecular oxygen with the release of hydrogen peroxide.

Fructosyl amino acid oxidases have been isolated from a number of different microorganisms, including bacteria,[15–17] filamentous fungi,[18–23] and marine yeast[24] (**Table 1**). Based on conserved primary structural features, such as FAD-binding motifs, all FAODs are members of the glucose-methanol-choline oxidoreductase family. Fructosyl amino acid oxidases can be subdivided according to origins and substrate specificity. Prokaryotic and eukaryotic FAODs form two structurally distinct groups, with very low homology between the two groups.[17]

Table 1.
Properties of Reported Fructosyl Amino Acid Oxidases

	Source	Molecular mass (kDa)	Substrate specificity group
Prokaryotic FAOD	*Corynebacterium* sp. 2-4-1[15,25,26]	44 (dimer)	*Group I* Prefer α-fructosyl amino acids (e.g., f-αVal)
	Agrobacterium tumefaciens AgaE-like protein[16]	42 (dimer)	
	Arthrobacter sp. FV1-1[17]	39 (dimer)	
Eukaryotic FAOD	*Aspergillus* sp. 1005[18]	43 (dimer)	
	Penicillium janthinellum AKU3413[19,20,27]	39–49 (monomer)	
	Ulocladium sp. JS-103[28]	—	
	Eupenicillium terrenum ATCC 18547[a] [21,29]	50 (monomer)	
	Coniochaeta sp. NISL 9330[a] [21,29]	52–60 (monomer)	
	Fusarium oxysporum S-1F4[30,31]	45–50 (monomer)	*Group II* Prefer ε-fructosyl amino acids (e.g., f-εLys)
	Fusarium oxysporum IFO-9972[32]	47–48 (monomer)	
	Aspergillus fumigatus Amadoriase I[21,33–35]	40–51 (monomer)	
	Aspergillus oryzae FAOD-A01[23]	39–49 (monomer)	
	Pichia sp. N1-1[24,36–38]	54 (monomer)	*Group III* React with both α- and ε-fructosyl amino acids
	Aspergillus fumigatus Amadoriase II[21,33,35,39]	49–55 (monomer)	
	Aspergillus oryzae FAOD-A02[23]	48 (dimer)	
	Aspergillus terreus GP1[20]	51 (dimer)	
	Pseudomonas sp.[b] [40,41]	106 (monomer)	

[a] These are FPOXs that also possess high activity toward α-fructosyl dipeptides (e.g., f-αVal-His).
[b] This FAOD has a copper prosthetic group instead of the FAD found in all other FAODs.

While the physiological role of eukaryotic FAODs remains unknown, extensive studies have led to a good understanding of the physiological role of prokaryotic FAOD as the key enzyme in the catabolic pathway of naturally occurring fructosyl amino acids. The most extensive studies have been carried out on the plant pathogen, *Rhizobium radiobacter* (*Agrobacterium tumefaciens*).[42–46] *R. radiobacter* causes crown gall tumors on higher plants by transferring discrete DNA fragments from its tumor-inducing plasmid to the nuclei of infected cells. Expression of the transferred genes results in the synthesis of opines, which are specifically utilized as nutrient sources by *R. radiobacter* that have genes for the appropriate opine catabolism systems. One of the opines existing in crown gall is fructosyl glutamine,[43] which is also called santhopine. Fructosyl amino acid oxidase serves as the key enzyme in the santhopine catabolic pathway. As the currently discovered prokaryotic FAODs from the genetically distinct bacteria *Corynebacterium*,[15,25] *R. radiobacter*,[16] and *Arthrobacter*[17] share very high homology, these highly conserved FAOD genes have been suggested to have been distributed by horizontal gene transfer at some time during their evolution.[17]

Prokaryotic FAODs are homodimers and possess a noncovalently bound FAD cofactor. Prokaryotic FAODs are specific for α-fructosyl amino acids, which are amino acids glycated on their α amino group. Most eukaryotic FAODs are monomers with a covalently attached FAD cofactor. Eukaryotic FAODs can be divided into three groups according to substrate specificity: those specific for α-fructosyl amino acids, those specific for ε-fructosyl amino acids, and those that oxidize both α- and ε-fructosyl amino acids at comparable rates (**Table 1**). Although all FAODs are unable to oxidize large glycated peptides or intact glycated proteins, some of the α-fructosyl amino acid-specific FAODs have recently been shown to have relatively high activity toward fructosyl valyl histidine (f-αVal-His),[21] which corresponds to the *N*-terminal fructosyl dipeptide derived from A1C. These enzymes are referred to as fructosyl peptide oxidase (FPOX).

Currently Available Enzyme-Based Technology for Glycated Protein Diagnosis

FAOD-based detection methods for glycated proteins have been commercially available since 1999 (**Table 2**).

Table 2.
Commercially Available Fructosyl-Amino-Acid-Oxidase-Based Assays for Measuring Glycated Proteins

Product	Manufacturer	Target molecule[a]	Measurement range[b]	FAOD used
CinQ HbA1c[47]	Arkray, Inc. (Japan)	A1C (f-αVal-His)	4.0–13.3%	FPOX
Norudia N HbA1c[48]	Sekisui Medical Co. (Japan)	A1C (f-αVal-His)	3.0–14.0%	FPOX
Direct Enzymatic A1C Assay[14,49]	Diazyme Laboratories (USA)	A1C (f-αVal)	4.0–16.0%	FAOD[c]
Lucica GA-L[50]	Asahi Kasei Pharma (Japan)	Glycated albumin (f-εLys)	3.2–68.1%	FAOD[d]
GlyPro Reagent[51]	Genzyme (USA)	Fructosamine (f-εLys)	3.5–1734 μmol/l	FAOD[d]

[a] The product liberated by proteolytic digestion that serves as substrate for FAOD is provided in parentheses.
[b] The measured A1C or glycated albumin measured is expressed as a percentage of total hemoglobin or albumin, respectively. The measured fructosamine (total glycated serum protein) is expressed as a concentration in μmol/l.
[c] This FAOD is reported as a f-αVal oxidase.
[d] This FAOD is reported as a ketoamine oxidase.

Because all FAODs are unable to react with intact glycated proteins, samples require an initial proteolytic digestion step to liberate glycated amino acids or glycated dipeptides. Current methods revolve around the same basic principle: (1) proteolytic digestion of samples, (2) FAOD reaction with liberated product, and (3) measurement of the resulting hydrogen peroxide using a peroxidase and a suitable chromogen.

Measurement of A1C is based on the measurement of the *N*-terminal fructosyl valine (f-αVal), while that of glycated albumin is based the measurement of fructosyl ε-lysine (f-εLys). It is important to measure only one of the two molecules according to the target protein. Many of these methods start with a separation step such as centrifugation; blood cells are used for the measurement of A1C, and serum is used for the measurement of glycated serum albumin. After proteolysis, an FAOD with an appropriate substrate specificity is used to help ensure that only molecules liberated from the target glycated protein are measured.

The CinQ HbA1c[47] and Norudia N HbA1c[48] systems use proteases that result in the liberation of the *N*-terminal glycated dipeptide f-αVal-His, which is then oxidized by an α-fructosyl amino acid-specific FPOX. The Direct Enzymatic A1C Assay[14,49] relies on extensive proteolytic digestion to liberate f-αVal, which is measured by an FAOD referred to as fructosyl valine oxidase by the manufacturer. This FAOD appears to be an α-fructosyl amino acid-specific FAOD, as test results are unaffected by the presence of glycated albumin. The Lucica GA-L[50] and GlyPro Reagent[51] systems determine the levels of glycated albumin and fructosamine, respectively, by employing an ε-specific FAOD to measure the f-εLys liberated from the protease digestion step.

The FAOD-based assay systems have been adapted to be carried out on automated analyzers, allowing the possibility to conveniently and rapidly measure a large number of samples. FAOD-based assays seem to have the reproducibility and accuracy of HPLC methods, with the convenience of immunoassay methods. Furthermore, unlike the immunoassay-based methods, FAOD-based methods have been shown to be unaffected by the presence of hemoglobin variants.[14]

Biomolecular Engineering of Fructosyl Amino Acid Oxidase

Fructosyl-Amino-Acid-Oxidase-Based Sensor Development

An additional advantage of using FAOD to monitor glycated protein levels is that it has the potential of being applied in an amperometric sensor. Such sensors seem ideally suited for creating a simple, convenient, and economical method of measuring glycated proteins for point-of-care treatment or self-monitoring applications.

Our group has been engaged in the development of a variety of molecules and principles for glycated protein biosensing.[44,52–55] Especially, we developed several electrode systems to measure f-αVal employing the

FAOD from the marine yeast *Pichia* N1-1[56–58] (**Figure 2**) and the soil bacterium *Arthrobacter* FV1-1.[59] The hydrogen peroxide sensor-based enzyme electrode and methoxy-5-methyl phenazinium methyl sulfate (mPMS) mediator-type enzyme sensor using carbon paste electrode exhibited good linear correlation.[56] To avoid, hopefully, the problems of applying a large potential, we also created amperometric enzyme sensors with low applied potential using a Prussian blue film as an artificial peroxidase as well as employing peroxidase and ferrocene as electron mediator.[57] The Prussian blue sensors not only avoided the inherent problems of measuring hydrogen peroxide with high applied potential, but also simplified the electrode construction compared to the two-enzyme construct. The hydrogen peroxide sensor-based enzyme electrode was also adapted to a flow-injection analysis system for measuring f-$^{\alpha}$Val.[58] The hydrogen peroxide sensor-based enzyme electrode was also constructed using the FAOD from the soil bacterium *Arthrobacter* FV1-1.[59]

Protein Engineering of Fructosyl Amino Acid Oxidase

Several groups have used protein-engineering approaches to improve the properties of FAOD. By carrying out random mutagenesis and screening for potentially useful mutants using an *in vivo* colorimetric plate assay, researchers succeeded in enhancing the substrate specificity of fungal FAODs. The substitution of a single amino acid residue in the FAOD from *Fusarium oxysporum* caused a great decrease in activity with f-$^{\alpha}$Val while only slightly affecting its activity with f-$^{\varepsilon}$Lys, thus greatly enhancing its f-$^{\varepsilon}$Lys specificity.[32] Similarly, substitution of one amino acid residue in the FAOD from *Ulocladium* sp. JS-103 resulted in a 14-fold greater preference for f-$^{\alpha}$Val compared to the wild-type enzyme.[28]

A different group also used random mutagenesis and *in vivo* colorimetric plate screening to improve FAOD thermostability. However, a directed evolution approach was used, whereby individual single mutations were combined to achieve cumulative improvements. By introducing a total of five mutations, the engineered *Corynebacterium* FAOD was stable at 45 °C, whereas the wild-type enzyme was unstable above 37 °C.[26] Using the same directed evolution approach, a total of six amino acid substitutions were introduced to greatly improve the thermostability of a fungal (*Coniochaeta* sp.) FAOD; however, the effects of the mutations on the enzyme's specific activity have not been reported.[60]

Despite the absence of any three-dimensional FAOD structural information, our group has adopted a rational design approach to improve enzyme properties. We set out to improve the substrate specificity of the FAOD from the marine yeast *Pichia* N1-1, which naturally reacts with f-$^{\alpha}$Val and f-$^{\varepsilon}$Lys at comparable rates. A three-dimensional structural model was created using as a template the crystal structure of a bacterial enzyme, *Bacillus* monomeric sarcosine oxidase, which shared significant similarities in primary structure.[36] Docking studies based on this model identified which residues interacted with the potential substrates f-$^{\alpha}$Val and f-$^{\varepsilon}$Lys (**Figure 3**).[37] Residue Asn354, which interacts closely with f-$^{\varepsilon}$Lys but not with f-$^{\alpha}$Val, was selected for site-directed

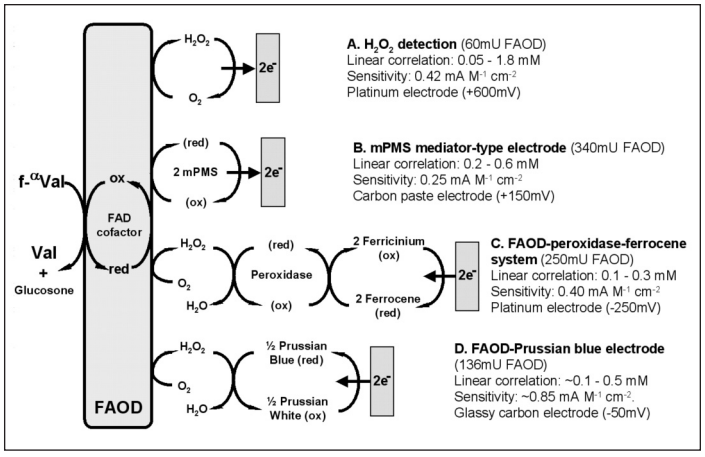

Figure 2. Principle behind various experimental f-$^{\alpha}$Val sensors using the fructosyl amino acid oxidase from *Pichia* N1-1 FAOD. The sensor systems are based on **(A)** hydrogen peroxide detection,[56] **(B)** mPMS mediator-type electrode,[56] **(C)** FAOD-peroxidase-ferrocene electrode system,[57] and **(D)** Prussian-blue-based FAOD electrode.[57] For each sensor, the units of FAOD used, the reported linear correlation and sensitivity, as well as the electrode composition and the applied potential are listed. The reduced and oxidized states of molecules are indicated with "red" and "ox," respectively.

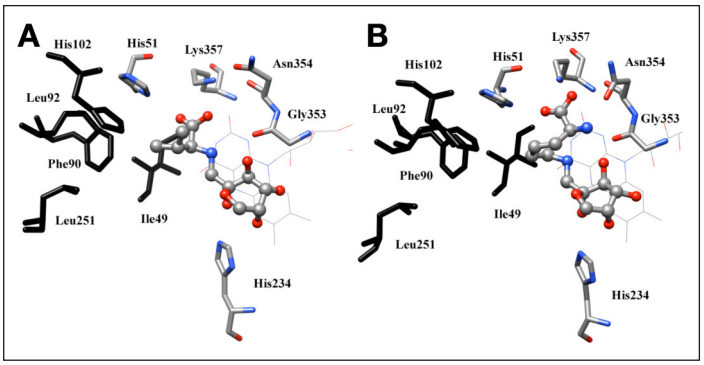

Figure 3. Docking models of **(A)** f-$^{\alpha}$Val and **(B)** f-$^{\varepsilon}$Lys at the active site of the *Pichia* N1-1 FAOD.[37] Hydrophobic residues are depicted in black, the FAD cofactor in thin lines, nitrogen atoms in blue, and oxygen atoms in red.

mutagenesis studies. Substitution of Asn354 to histidine and lysine simultaneously increased the enzyme's activity toward f-$^{\alpha}$Val and decreased that toward f-$^{\varepsilon}$Lys, thus greatly improving its specificity for f-$^{\alpha}$Val (**Table 3**). Substitution of residue His51 also produced mutants with significantly improved specificity for f-$^{\alpha}$Val (**Table 3**).[38] A cumulative effect was observed by combining the As354 and His51 amino acid substitutions, producing an FAOD mutant with greatly improved f-$^{\alpha}$Val specificity.

Future Direction

The three-dimensional structure of an FAOD (Amadoriase II) from the fungus, *Aspergillus fumigates*, was recently solved in the free and inhibitor-bound form.[39] This crystal structure supports our predicted structural model and validates our rational design approach. This is expected to greatly contribute to future rational designing of any FAOD, providing useful information that can be applied to further improve stability, substrate specificity, or properties for specific sensor applications. The crystal structure identified a 12 Å deep catalytic site, providing a possible explanation for the inability of FAOD to oxidize large glycated peptides.[39] Continued protein engineering investigations may lead to an increase in the size of acceptable substrates to hopefully eventually engineer an FAOD that is able to accept intact glycated proteins, thus making the proteolytic digestion unnecessary.

Fructosyl-amino-acid-oxidase-based sensors will be developed using the currently available enzymes together with technologies that have been well established for the self-monitoring of blood glucose. The combination of bioengineering approaches in diagnostic systems and the biomolecular engineering of FAOD can lead to the development of an accurate, rapid, convenient, and economical glycated protein biosensing system suitable for point-of-care treatment and personal use.

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Table 3.
Kinetic Parameters of Purified Wild-Type and Mutant Fructosyl Amino Acid Oxidases

	f-$^{\alpha}$Val			f-$^{\varepsilon}$Lys			B/A
	K_m (mM)	V_{max} (U mg^{-1})	$V_{max}K_m^{-1}$ (A) (U mg^{-1} mM^{-1})	K_m (mM)	V_{max} (U mg^{-1})	$V_{max}K_m^{-1}$ (B) (U mg^{-1} mM^{-1})	
Wild type	3.9	6.5	1.7	0.9	9.5	11	6.5
Asn354His[37]	0.6	16	27	3.9	16	4.1	0.15
Asn354Lys[37]	2.0	25	13	15	21	1.4	0.11
His51Lys[38]	2.4	10	4.2	2.0	15	7.5	1.8
His51Arg[38]	11	17	1.5	18	8.5	0.47	0.31
His51Lys/Asn354His[38]	0.4	11	28	27	33	1.2	0.043
His51Arg/Asn354His[38]	2.4	15	6.3	—[a]	—[a]	—[a]	—[a]

[a] Not determined.

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