SMALL ANGLE NEUTRON SCATTERING REVEALS DIMERIC GLUCOSE OXIDASE FROM *ASPERGILLUS NIGER* AT pH 5.9

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Abstract – Glucose oxidase (GOx) is a 160 kDa flavoenzyme dimer belonging to the family of glucose-methanol-choline oxidoreductases. Despite the abundant availability of information regarding the structure and mechanisms of interactions of GOx, there is still considerable interest in studying the properties of this protein to extend its bio-applications. The present study aims at investigating the conformational stability of GOx from *Aspergillus niger* in the optimal environment that presumably preserves its functional properties using small angle neutron scattering method. This method allowed computing the low-resolution three-dimensional models of the protein. Obtained results indicate protein dimerization in buffer solution pH 5.9, with a maximum particle dimension, D_{max} of 110 Å and R_g of 34.50±0.025 Å.

Keywords: glucose oxidase, conformation in solution, dimer, small angle neutron scattering

INTRODUCTION

Flavoproteins are a class of enzymes that catalyze various chemical reactions through their non-covalently bonded substrates flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Flavoproteins participate in reactions of electron transfer and dehydrogenation. Also, they are actively involved in a variety of physiological processes including protein folding, DNA repair, oxygenation, various redox reactions, detoxification, neural development, *etc* [1]. These proteins are extensively studied due to their physicochemical properties that are of interest for various pharmaceutical, food industries and more recently,

medical applications [1-5]. One of the most studied flavoproteins is glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase) [2,3].

Glucose oxidase (GOx) is a dimeric enzyme (Fig. 1a), mainly known as catalyst of the oxidation reaction of β-D-glucose to glucono-β-lactone and H_2O_2, using molecular oxygen (Fig. 1c). It is a member of the glucose-methanol-choline oxidoreductases family. The GOx monomer (Fig. 1b) with a molecular weight of 80 kDa consists of a single polypeptide chain of 583 amino acids that are organized mostly in α-helix and β-sheets structures [2]. The monomer chain is folded into two domains and has one non-covalently bound molecule of FAD (the inset in Fig. 1b).

The catalytic activity of glucose oxidase and its bio-applications are mainly limited by the conformational stability. So far, it is known that transitions from α-helix to β-sheets secondary structures or complete unfolding of the protein will result in release of FAD and loss of enzymatic activity [3]. Intrinsic changes such as subtle local conformational transitions of GOx are induced by buffer solutions and salt concentration, whilst the extrinsic changes resulting in conformational changes are highly dependent on pH and temperature of the protein environment. The solution pH is relevant in preserving the proper protein conformation as it was shown that the optimum pH for GOx ranges between 5 to 6 [4]. Temperature and substrate concentration are also key factors defining the reactivity of GOx. The optimum temperature reported for GOx ranges between 30 and 50°C, higher temperatures induce denaturation of FAD substrate and result in enzyme inactivation [5].

The purpose of the present study is therefore to ascertain the conformational stability of GOx from *Aspergillus niger* in the optimal environment that presumably preserves its functional properties using small angle neutron scattering (SANS) method. SANS is an efficient method to study the overall protein conformation in solution from *ab initio* analysis and rigid-body modelling and compare the low resolution models of solution structures to high-resolution models of the crystal structure [6-8]. Thus, obtained results enabled (i) characterization of GOx conformation in solution and (ii) determination of its oligomeric state based on solution scattering-derived parameters.

EXPERIMENTAL

Sample preparation

All reagents used in this study were purchased (Sigma-Aldrich) and used without any further purification. The lyophilized powder of GOx from *Aspergillus niger* was dissolved in

50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer in deuterated water, pH=pD=5.9 [8]. The concentration of GOx was fixed at 15 mg/mL. No salt was added to the studied system, since it is already known that in the presence of NaCl, GOx undergoes local conformational changes in the oxygen active site [2].

SANS measurement

SANS experiment was carried out using the KWS-2 diffractometer operated by the Jülich Centre for Neutron Science Outstation at Heinz Maier-Leibnitz Zentrum Garching, Germany [9]. The scattering profile was recorded at a fixed neutron wavelength λ = 5 Å. The sample was measured in quartz Helma cell of 1 mm path length, at the temperature 293 K. Data collected from two sample-to-detector distances (1.16 and 7.61 m) were merged using dedicated software QtiKWS to obtain a complete scattering profile for the analyzed system in the 0.0076 to 0.46 Å-1 *Q*-range. SANS data analysis and *ab initio* reconstruction were performed using ATSAS vs. 2.7.1 software package [10]. High resolution models 3qvr.pdb, 1gpe.pdb and 4moe.pdb were used to obtain the theoretical small-angle neutron scattering profiles as well as R_g for monomeric, dimeric and tetrameric forms of protein using CRYSON from ATSAS vs. 2.7.1 software package.

All graphical representations were generated using VMD software [11].

RESULTS AND DISCUSSION

Characterizing the GOx

This study was designated to assess the conformation stability of GOx from *Aspergillus niger* in the environment where supposedly it will preserve its functional properties using SANS technique. In a SANS study, the scattered intensity for a monodisperse system is described as $I(Q)=(N/V)Vp^2(\Delta\rho)^2P(Q)S(Q)$, Q is the scattering vector, $Q=4\pi(\sin\theta)/\lambda$, where 2θ is the scattering angle; φ is the volume fraction of the scattering object; Vp is the volume of a scattering molecule; $\Delta\rho$ is the difference of scattering length density between the scattering molecule and the solvent; $P(Q)$ is a square of form factor $(P(Q) = |F(Q)|^2)$ that is the scattering of a single molecule describing the shape and size of individual scattering molecule; $S(Q)$ is the structure factor, describing the interactions and distribution of scattering molecules in solution [12]. GOx in solution mainly exists in dimeric form, however, the theoretical scattering intensities were calculated using the atomic coordinates of GOx crystallographic structures of monomeric, dimeric and tetrameric form (PDB entry 3qvr, 1gpe and 4moe, respectively),

considering 100% D_2O fraction in solvent. Predicted values of the small angle neutron scattering structural parameters estimated for all three forms of GOx are presented in Table 1.

Experimental scattering curve, without any artificial truncation at low Q obtained after buffer subtraction is shown in Fig. 2. The downward registered at low Q is an indicative of interparticles associative interactions occurring in the studied system [6]. To obtain the basic structural information from the experimental scattering curve and compare to predicted values, a series of standard plots was constructed.

Guinier approximation, represented as $I(Q)=I(0)exp[-(QRg)^{2/3}]$, was used to determine the values of radius of gyration R_g and forward scattering intensity $I(0)$. The linear Guinier plot $(ln[I(Q)])$ versus Q^2 obtained for the $0.029<Q<0.052$ Å^{-1} region (the inset in Fig. 2a) yielded an R_g value of 34.48±0.16 Å that is close to the predicted R_g value of dimeric form of GOx and an $I(0)$ of 2.31±0.02 cm^{-1}.

Fig. 2a depicts theoretical neutron scattering profiles of GOx fitted to the experimental curve in the 0.027 to 0.302 Å^{-1} Q-range that for comparison purposes are presented along with the experimental scattering curve. According to Fig. 2a and the predicted scattering parameters included in Table 2, theoretical scattering profile corresponding to dimeric conformation seems to reproduce better the experimental scattering curve in comparison to the monomeric and tetrameric form.

The data reported in the form of Kratky plot ($Q^2I(Q)$ versus Q) show that GOx in the studied system is completely folded (see the inset in Fig. 2b), but not globular because Kratky plot presents a peak at Q=0.05 Å^{-1}, QR_g=1.73 instead of QR_g=1.2 as it is expected for globular proteins [13]. As the SANS study was performed on systems containing GOx in solution pH 5.9 without the addition of a stabilizer, analysis of the flexibility that contributes to the dynamics of the protein was performed. The intrinsic flexibility of the protein structure was assessed using the Porod-Debye plot ($Q^4I(Q)$ versus Q^4) that in case of non-flexible proteins it should contain a plateau within the low to intermediate Q-region that disappears with the increase of the protein flexibility [14]. The dimensionless Porod-Debye plot obtained for 0.008-0.097 Å^{-1} Q-region, presented in Fig. 2c is showing a plateau consistent with folded protein with little flexibility. A reduced flexibility of protein structure is induced by the stable dimeric structure of GOx.

The scattering parameters were also computed using the indirect Fourier transform analysis from GNOM program (ATSAS vs. 2.7.1) on the extended angular range 0.027 Å^{-1} <Q<0.302 Å^{-1}. The obtained pair-wise distribution function $P(r)$ showed a profile that usually is attributed to slightly elongated shapes, with a maximum particle dimension, D_{max} of 110 Å

(total estimate from GNOM, X^2= 0.751). The values of R_g= 34.50±0.02 Å and *I(0)*= 2.29±0.02 cm^{-1} are in close agreement with those extracted using Guinier approximation.

GOx shape and sizes

The low resolution models of GOx molecular envelope were calculated using two *ab initio* computational approaches, dummy atoms and dummy residues. Albeit, both methods do not allow the differentiation of secondary structure elements, it is still possible to portray the overall shape of the protein molecule. The dummy atoms (Fig. 3a) and dummy residues (Fig. 3b) models were built using the pair-wise distribution function *P(r)* (Fig. 3c) and a 2-point symmetry (P2) constraint in the calculations. This assumption was supported by the scattering parameters derived from experimental and modelled theoretical scattering curves pointing that all GOx molecules in buffer solution, pH 5.9 have the shape of dimeric structures.

The dummy atoms model calculations were performed using the angular range $Qmax \approx 8/R_g$, as it is recommended for usage of DAMMIF program (ATSAS vs. 2.7.1). The best dummy atom model along with the fit of the experimental scattering data is presented in Fig. 3a. The model was selected based on the lowest normalized spatial discrepancy (NSD) [15], which is computed using SUPCOMB program (ATSAS vs. 2.7.1) after superimposing onto the crystallographic model of GOx dimer (1gpe.pdb). The dummy molecular model presented in Fig. 3a contains 4392 dummy atoms with a radius of 2.00 Å. The NSD between the high resolution dimeric model 1gpe.pdb and dummy atom envelope is 1.98.

The *ab initio* dummy residue models were calculated using GASBOR program (ATSAS vs. 2.7.1). Multiple runs were performed varying the angular range of the scattering data from 0.027 $Å^{-1} < Q < 0.302$ $Å^{-1}$ to 0.027 $Å^{-1} < Q < 0.210$ $Å^{-1}$, these gave almost coincident results. The best dummy residue model was also selected based on the lowest NSD value. Fig. 3b shows the dummy residue model and the fit of scattering data. The model comprises 2154 dummy atoms with a radius of 1.9 Å (NSD= 2.49). It presents very good resemblance with the dummy atom model and the crystallographic structure of GOx dimer (Fig. 3). The dummy residue dimer has a similar overall shape that is possible to segment into two units with consistent shape to the monomer.

The low resolution model built using dummy residue approach with the approximate dimensions of 84×67×29 Å is slightly more elongated when comparing to 77×60×39 Å of the high resolution model (Fig. 3 b,c). Taking into account the flexibility and conformational changes that the protein molecules undergo in solution, the NSD values calculated for both,

dummy atoms and dummy residues, can be considered reasonably good, confirming GOx dimerization in solution.

CONCLUSIONS

Conformational stability of biological macromolecules is one of the major problems for biotechnology, pharmaceutical and food industry. Defining a technique suitable for studying the protein conformation is essential for optimizing the properties of proteins. Although considerable research has been devoted to the structure, conformation stability and mechanisms of interactions of glucose oxidase (GOx), there is still a continuous interest in studying the properties of this protein to extend its bio-applications. The work presented here was addressed to the study of GOx conformational stability in solution. The functional oligomeric state of GOx is a dimer, although evidence from the high-resolution X-ray crystallographic structures of the monomeric and tetrameric forms is also available (PDB entry 3qvr, 1gpe and 4moe). Using SANS it was shown that at pH 5.9 in HEPES buffer solution of 100% D_2O and a concentration of 15 mg/mL, GOx exists in a dimeric state. Also, the low-resolution three-dimensional models of GOx from *Aspergillus niger* was computed based on SANS results. The neutron scattering results indicate that GOx model adopts a slightly more relaxed structure in solution comparing to the compact X-ray high resolution model with a maximum particle dimension, D_{max} of 110 Å and R_g of 34.50±0.02 Å.

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Figure captions.

Fig. 1. Graphical representation of GOx. (a) The overall structure of the GOx dimer is presented as surface (PDB entry 1gpe) and (b) its monomeric form as cartoon (3qvr.pdb) with an inset showing the close-up view of the flavin adenine dinucleotide and the binding pocket. (c) The oxidation reaction scheme of β-D-glucose catalyzed by glucose oxidase.

Fig. 2. Solution neutron scattering results for GOx protein. (a) Comparison of the experimental (15 mg/mL GOx in buffer 100% D_2O) and the modelled scattering pattern for monomeric,

dimeric and tetrameric form of GOx using the program CRYSON. The inset presents the Guinier region showing the linear regression fit of the experimental data. (b) The Kratky plot obtained for the experimental curve shows that the protein is folded though not globular as it has a peak at Q=0.05 Å^{-1} (QR_g = 1.73). (c) The dimensionless Porod-Debye plot showing a plateau at low-Q consistent with folded protein with little flexibility.

Fig. 3. The *ab initio* model calculations based on SANS data. (a) The fit of scattering data and dummy atoms molecular envelope resulted from DAMMIF calculations. The normalized spatial discrepancy (NSD) between the high resolution dimeric model 1gpe.pdb and the envelope is 1.9826. (b) GASBOR dummy residue fit of the scattering data and modelled molecular envelope (NSD= 2.4853). (c) The pair-wise distribution function *P(r)* of GOx shows a maximum dimension of 110 Å, the inset showing the fit of scattering computed for final *P(r)* function to experimental data and the dummy residue envelope superimposed onto cartoon representation of high resolution dimeric GOx, 1gpe.pdb.

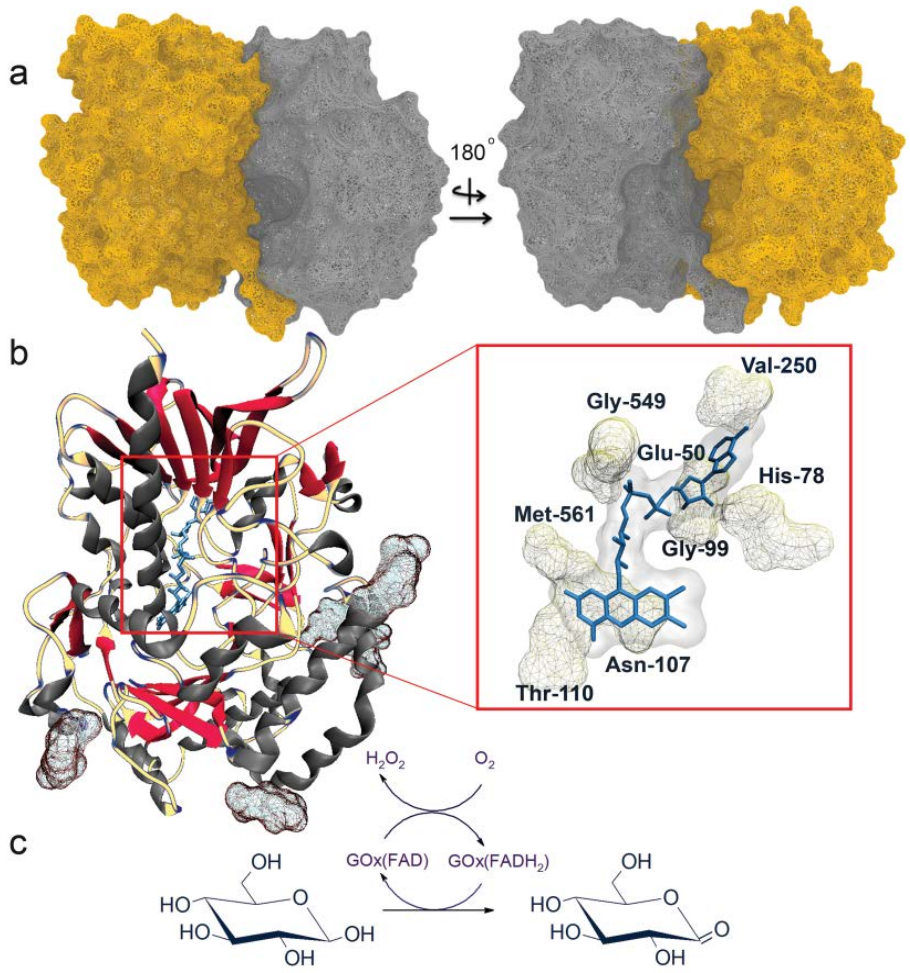

Fig. 1. R.V. Erhan, *Journal of Surface Investigations*

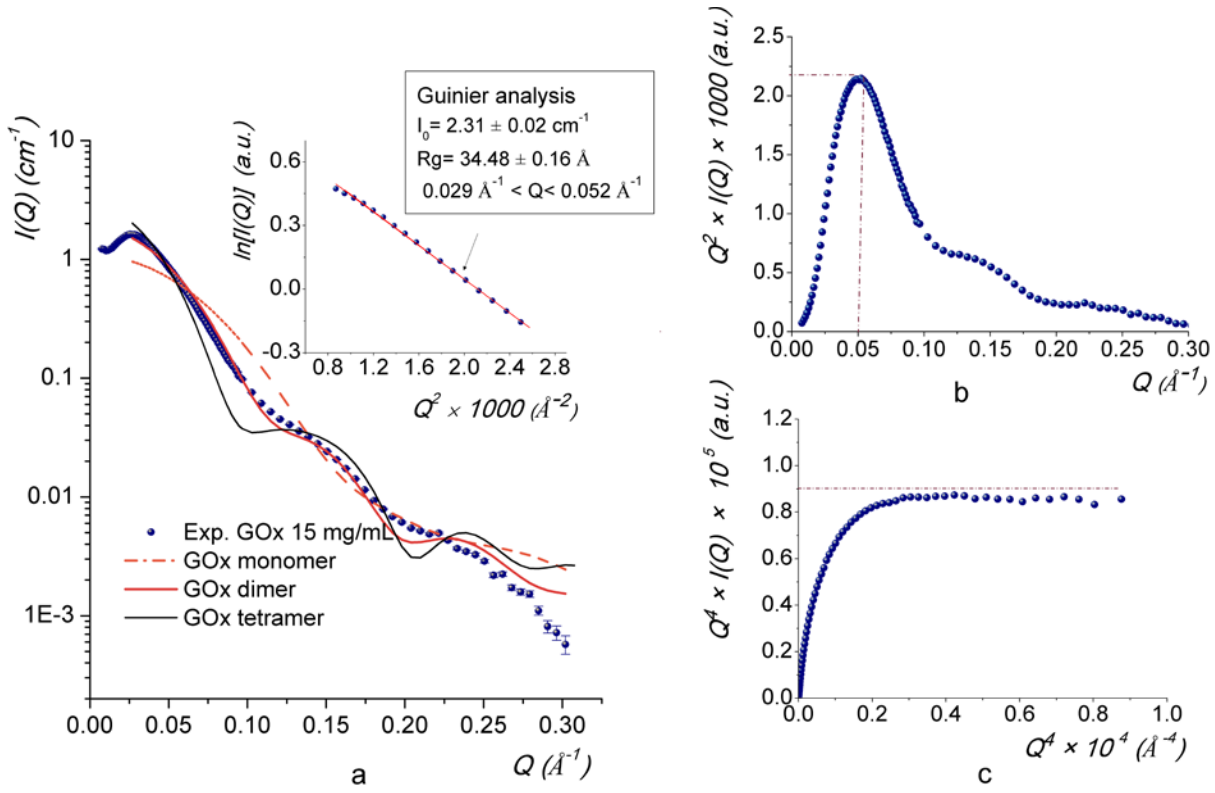

Fig. 2. R.V. Erhan, *Journal of Surface Investigations*

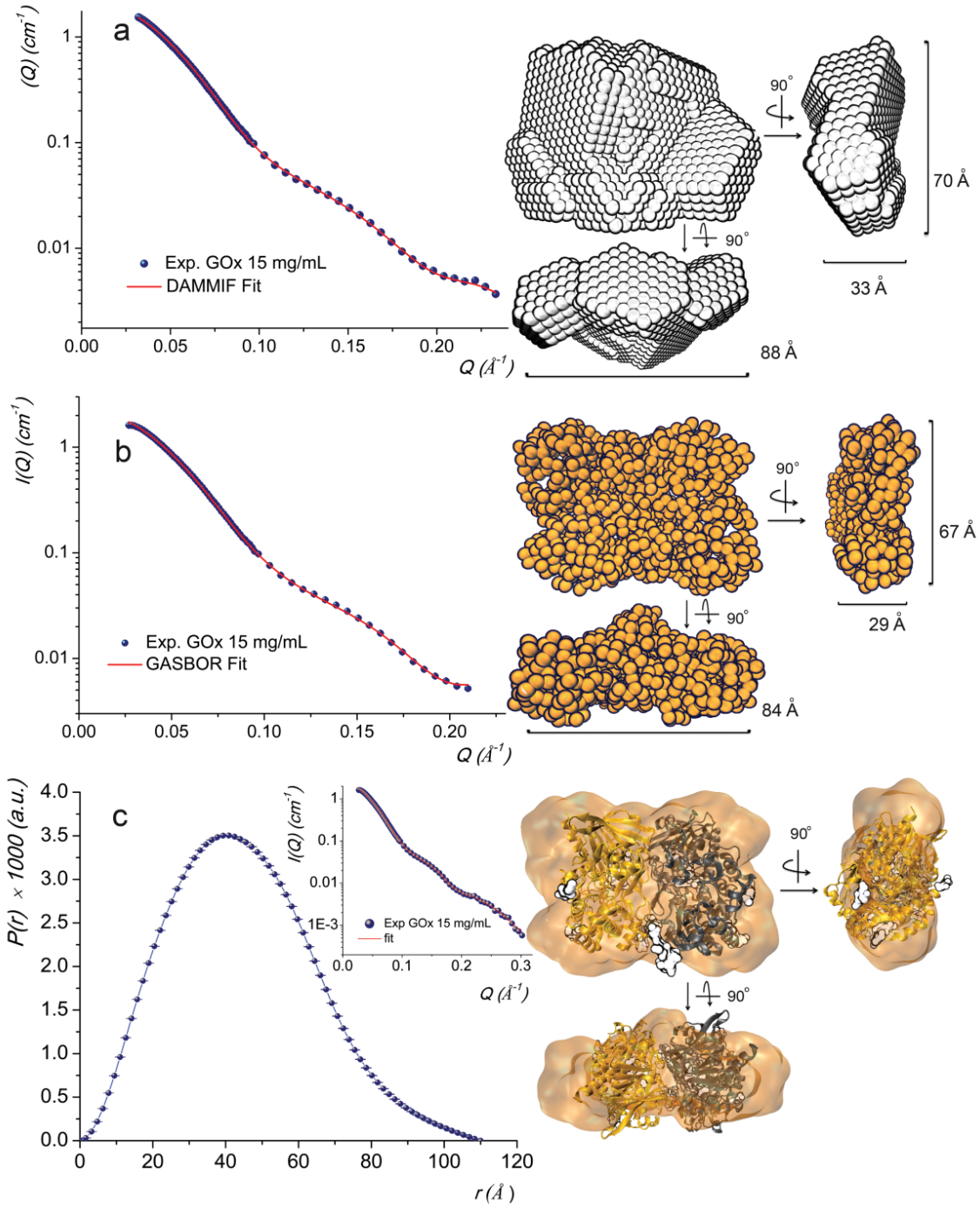

Fig. 3. R.V. Erhan, *Journal of Surface Investigations*

Table 1. Data collection and overall scattering-derived parameters for glucose oxidase.

Data collection parameters			
Instrument	KWS-2 small angle neutron scattering diffractometer		
Wavelength (Å)	5		
Q range (Å^{-1})	0.0076 - 0.46		
Total exposure time (min)	40		
Temperature (K)	293		
Predicted structural parameters			
GOx conformation	Monomer	Dimer	Tetramer
PDB entry	3qvr	1gpe	4moe
Atomic R_g (Å)	23.59	30.10	39.84
Envelope R_g (Å)	23.53	29.84	39.27
Envelope diameter (Å)	80.14	95.37	131.9
Molecular weight (kDa)	65.75	132.7	262.9
Dry volume (Å^3)	~79690	~160800	~318600
Structural parameters			
I(0) (cm^{-1}), from Guinier	2.31±0.02		
R_g (Å), from Guinier	34.48±0.16		
I(0) (cm^{-1}), from *P(r)* (X^{2a}= *0.751)*	2.29±0.02		
R_g (Å), from *P(r)* (X^{2a}= *0.751)*	34.50±0.02		
D_{max} estimate (Å)	110		
Porod volume estimate (Å^3)	~167590		

[a] *total estimate from GNOM.*