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Crystal structure of apo-glycolate oxidase

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The crystal structure of the apoform of the α/β-barrel enzyme glycolate oxidase has been determined to 2.6 Å resolution. Removal of the tightly bound cofactor FMN has a very strong influence on the protein structure; it is converted into a very flexible state, verging on a molten globule type of structure. The asymmetric unit contains two subunits with different conformations to each other and to the holo-enzyme. The secondary structures are preserved, but their mutual arrangement has changed to some extent introducing cavities into the protein. The largest structural shifts are, however, found in the loops.

Glycolate oxidase; Apostructure; Protein crystallography; Flavin binding

1. INTRODUCTION

Glycolate oxidase (glycolate:oxygen oxidoreductase, EC 1.1.3.1; GOX) is a peroxisomal flavoenzyme that catalyses the oxidation of α-hydroxy acids to the corresponding α-ketoacids. In green plants, GOX is one of the enzymes in photorespiration, a pathway that results in reduced net photosynthesis. In animals, the enzyme participates in production of oxalate. Inhibition of GOX might be important, in plants to increase net photosynthesis and in animals to treat diseases caused by calcium oxalate overproduction.

Pure enzyme has been prepared from different plants [1,2] and the liver of different vertebrates including human liver [3,4]. GOX molecules are present in solution as tetramers and/or octamers of identical subunits of 43,000 Da [1,4]. The amino acid sequence of spinach GOX is known [5,6] and the three-dimensional structure of the protein has been determined and refined to 2 Å [7].

GOX belongs to the flavoprotein family that has an α/β barrel structure. Besides GOX, two other closely related flavoproteins, flavocytochrome b_2 [8] and trimethylamine dehydrogenase [9] have the same fold [10]. Crystallographic studies have shown that the FMN molecule is bound in a similar way by all these proteins, at the carboxyl end of the β-strands in the barrel. The coenzyme is almost entirely buried inside the protein and forms extensive interactions with protein atoms. All residues that form hydrogen bonds to the flavin are C-terminal residues of the β-strands or are located in the loops which join the β-strands and subsequent α-helices.

Coenzyme binding to proteins often results in increased stability and changes of some properties of the enzyme which is usually explained by enzyme conformational transitions. For some coenzymes, structural information of their influence on the three-dimensional structure of the enzyme is available, e.g. NAD(P) binding to dehydrogenases has been studied in detail [11,12]. It was shown that upon binding of the coenzyme, alcohol dehydrogenases undergo a large conformational change: the catalytic domain makes a large rigid-body rotation towards the coenzyme-binding domain. Similar studies have been performed for $NADP^+$ in dihydrofolate reductase [13], the thiamin diphosphate dependent enzyme transketolase [14], and other enzymes [15].

There is a considerable amount of indirect evidence for a strong influence of flavin association on the protein structure [16,17] but only in one case, glutathione reductase [18] a structural investigation has been performed. No information is available on the influence of FMN on the structure of a flavoprotein which consists of an α/β barrel. We have therefore undertaken a structure determination of the apoform of GOX to analyze the influence of the cofactor on the three-dimensional structure of the enzyme.

2. EXPERIMENTAL

2.1. *Data collection*

GOX was prepared from fresh spinach as described earlier [19]. Under conditions of very high salt concentration, FMN dissociates from the enzyme. Crystals of the apoform of GOX were obtained using ammonium sulfate as precipitating agent as described earlier [19]. The crystals belong to spacegroup P42,2 with cell dimensions $a = b = 145.5$ Å and $c = 103.5$ Å. Packing considerations showed that

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the asymmetric unit must contain at least two independent subunits. A data set to 2.6 Å resolution using 7 crystals (70% complete, *R*-merge = 10.4%) was collected on film on a Rigaku rotating anode. Films were processed with the OSC program [20,21] and the data were scaled [22] and processed with the CCP4 program package (Daresbury, UK).

2.2. *Molecular replacement*

The ALMN rotation function program [23] was used throughout. Initial runs of the selfrotation function did not give rise to any peaks above background. Structure factors were calculated for a model of holo-GOX inserted into a large unit cell and the cross rotation function was run for different inner and outer integration radii and in different resolution shells. Only one consistent independent peak, $\alpha = 52°$, $\beta = 10°$, $\gamma = -65°$, could be found. However, calculation of the native Patterson function gave a large peak, 1/4 of the origin peak at 0, 0, 0.51. The two subunits are thus related by a translation along the *c*-axis of about 53 Å. After application of the rotation obtained, translation function calculations (program TFSGEN written by Ian Tickle in the CCP4 program package) gave one peak at 55.5 Å, 9.7 Å and 27.2 Å, height 5962. The next highest peak, 55.5 Å, 9.7 Å, 0.9 Å had height 3905. As a check of this solution we soaked a crystal with $K_2Hg(Cl)_4$, which in case of the holo-enzyme had given a good single site derivative. An 8 Å resolution data set was collected on a STOE diffractometer and the solution of the resulting difference Patterson was consistent with the result from the rotation function and the highest peak in the translation function. We could thus translate the two model subunits into the correct positions in the cell.

2.3. *Refinement of model*

Rigid body refinement using CORELS [24] was performed on each subunit yielding an *R*-factor of 47.4%. The model obtained was then subjected to rigid body refinement using the program XPLOR [25]. In this case each secondary structure and loop was treated as a rigid body. This decreased the *R*-factor to 43.3% at 3.0 Å. We then ran simulated annealing refinement. A total of 23,209 reflections corresponding to a resolution of 2.6 Å were used. After each round the model was compared with newly calculated $|F_o|-|F_c|$ and $2|F_o|-|F_c|$ difference Fourier maps and in some cases omit maps. Mispositioned residues and incorrect conformations were adjusted using the program 'O' [26]. After the first round, water molecules were incorporated at places where the density in $|F_o|-|F_c|$ map was higher than 2.5 σ and if they fulfilled the geometrical requirements for hydrogen bond formation. After each round, all solvent molecules were inspected and those having very low densities were deleted.

The refinement protocol consisted of a molecular dynamics simulation where the temperature was raised to 4,000K and then slowly cooled to 300K in steps of 50K, followed by 80 steps of energy minimization. Repeated rounds of refinement, in the end including individual *B*-factors, resulted in an *R*-factor for the model of 22.6%. The main-chain dihedral angles were not restrained during refinement and about 15 non-glycine residues for each subunit were found in energetically unfavorable regions in a Ramachandran plot.

In the final model, root-mean-square (rms) bond length and bond angle deviations are 0.022 Å and 4.6°, the rms deviation in dihedral angles is 27.1° and rms in improper angles is 1.8°.

2.4. *Structure analysis*

The structure has been analysed using 'O' [26]. The two subunits were superimposed on each other and on holo-GOX using the LSQ-option. The program VOIDOO (Kleywegt, G. and Jones, T.A., unpublished) was used to calculate volume of the molecules and to search for cavities inside the subunits. In calculating the volume some residues of apoenzyme were deleted and flavin was removed from the holo-enzyme to make the number of atoms equal in all molecules. XPLOR and DSSP [27] were used to calculate accessible surface area (probe radius for solvent was 1.4 Å). The schematic picture of GOX was drawn using Molscript [28].

3. RESULTS AND DISCUSSION

3.1. *Electron density map*

The refined model of apo-GOX comprises 357 residues from subunit 1 and 354 residues of subunit 2 and in total 197 water molecules. Some of these water molecules might be sulfate ions because the crystals were grown at high concentration of sulfate. In the native GOX structure there is a flexible loop (residues 189–197). In the apo-enzyme, parts of this loop have a more defined conformation and can be identified in their electron density, but there is no electron density for residues 192–194 in subunit 1, and residues 191–195 in subunit 2. Another loop which contains residues that are part of the active site, the loop between strand 6 and helix 6 (residues 253–261) is very poorly defined in the second subunit. Some surface exposed sidechains are also poorly defined. At a cut-off level of the standard deviation of the electron density map, no density is observed for flavin, which confirms that the crystals do not contain any bound flavin and the protein is the apoform of GOX. This is also supported by the fact that the crystals of apo-GOX are, in contrast to the yellow holo-enzyme crystals, colourless.

3.2. *Structure of apo-enzyme*

Fig. 1 shows the schematic structure of native GOX with labels on helices and β-strands as referred to in the text. The asymmetric unit of apo-enzyme crystals contains two subunits of apo-glycolate oxidase. Their structures differ from each other and from the holo-enzyme (Fig. 2): all C_α atoms superimpose with an rms value of 1.3 Å, 1.2 Å, and 1.6 Å for pairs apo-subunit 1–GOX, apo-subunit 2–GOX, and two subunits of apo-enzyme, respectively. These are rather large numbers for identical subunits. The superposition of holo-GOX and one domain of flavocytochrome b_2 from yeast which have 37% sequence identity yields an rms difference of 0.96 Å for 311 C_α-atoms [10]. The dissimilarities in structure between apo- and holo-GOX is due to differences in the mutual arrangement of β-strands and α-helices and especially in loops in the two subunits and in comparison with holo-GOX. Differences in crystal packing for the two subunits result in shifted contact areas of neighboring subunits and is partly responsible for the distinction in structure.

Without cofactor the volume of the subunit increases from 34,960 Å^3 (holo-GOX) to 35,690 Å^3 (subunit 1) and to 35,740 Å^3 (subunit 2). The volume increase is due to the appearance of cavities rather than a general swelling. Holo-GOX is a rather solid subunit: there are no cavities inside available to solvent molecules (probe radius for water was 1.4 Å) and only 5 small cavities with total volume 154 Å^3 which are not occupied by protein atoms. The funnel-shaped active site is accessible for the bulk solution and cannot be considered as a cavity. The space which is not occupied by protein atoms inside the

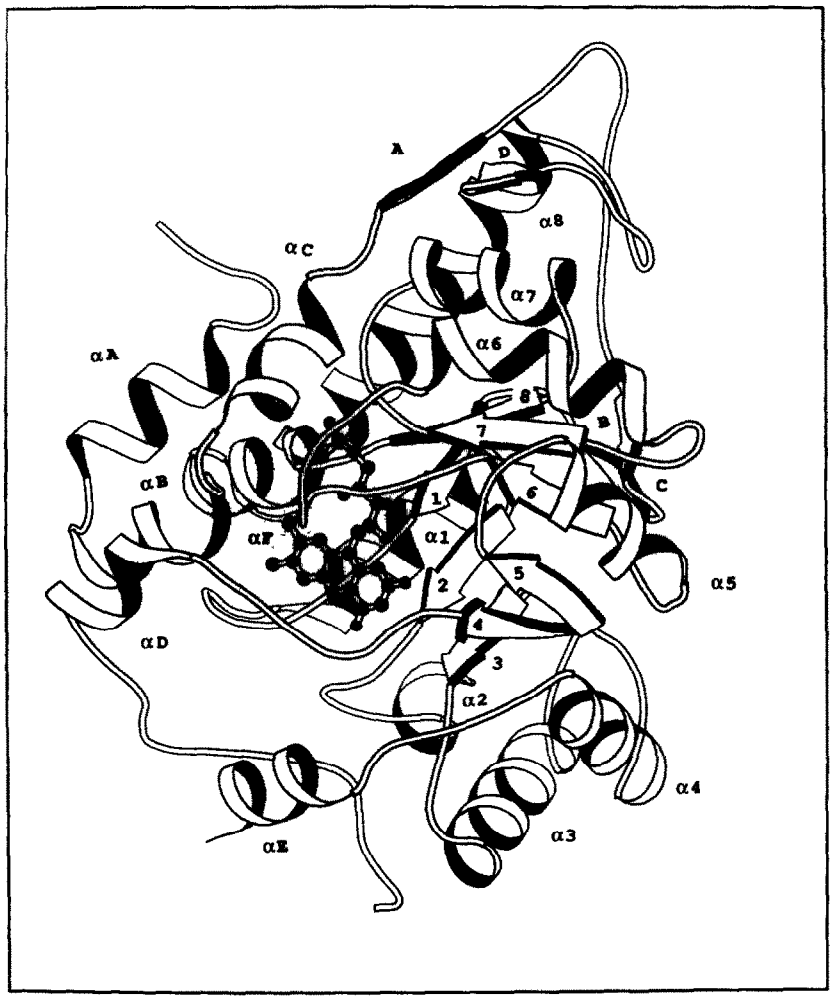

Fig. 1. Schematic drawing of holo-glycolate oxidase.

apo-enzyme is much larger: VOIDOO found 11 cavities in each subunit with a total volume of 916 Å^3 and 1,236 Å^3, respectively. Most of these cavities are small but one of them, in subunit 1, has a volume 157 Å^3 and in the second subunit there are two large cavities (90 Å^3 and 310 Å^3). The surface area accessible to solvent has increased for apo-monomers compared to holo-GOX, from 13,800 Å^2 to 14,750 Å^2 and 14,600 Å^2, respectively. The number of hydrogen bonds is retained in subunit 2 and decreased slightly (by 7 out of 248 in total) in subunit 1. The average B-factor of the apo-enzyme is of the same order or slightly higher (27.1 Å^2 for GOX, 29.5 Å^2 and 28.1 Å^2 for apo-enzyme). All these data indicate that the apo-enzyme is much more flexible and not so compact as holo-GOX.

This flexibility does not disrupt the general fold of the domain and although FMN is bound at the C-terminus of the β-strands the core of the barrel is quite undisturbed: rms deviations for 43 superimposed C_α atoms of residues forming the parallel β-strands are about 0.6 Å and the barrel is the most unperturbed region of glycolate oxidase. Even side chains of those residues that formed hydrogen bonds with FMN atoms change less than residues from loop regions, even when they are far from bound FMN.

The movements of helices after flavin dissociation are larger. These shifts give rise to the above mentioned cavities. The cavity in subunit 1 is located near Pro-77 between helix 2 and residues from the loop joining strands B and C opposite to the isoalloxazine binding site. Helices 1 and 2 come closer to each other and close the region from solvent. Two water molecules are located inside this cavity. The largest cavity in subunit 2 is located between helix 8 and strand D. The second cavity in subunit 2 is near the ribityl binding site close to helix C and loops 6 and 7. Flavin dissociation causes not only movement of helices as rigid bodies but in a few cases the helices start to unwind at their ends and the long helix 8 becomes bent.

Loops have the most divergent conformations among the three monomers (Fig. 2) but loops at the N-terminal end of the barrel joining a helix and β-strand differ less

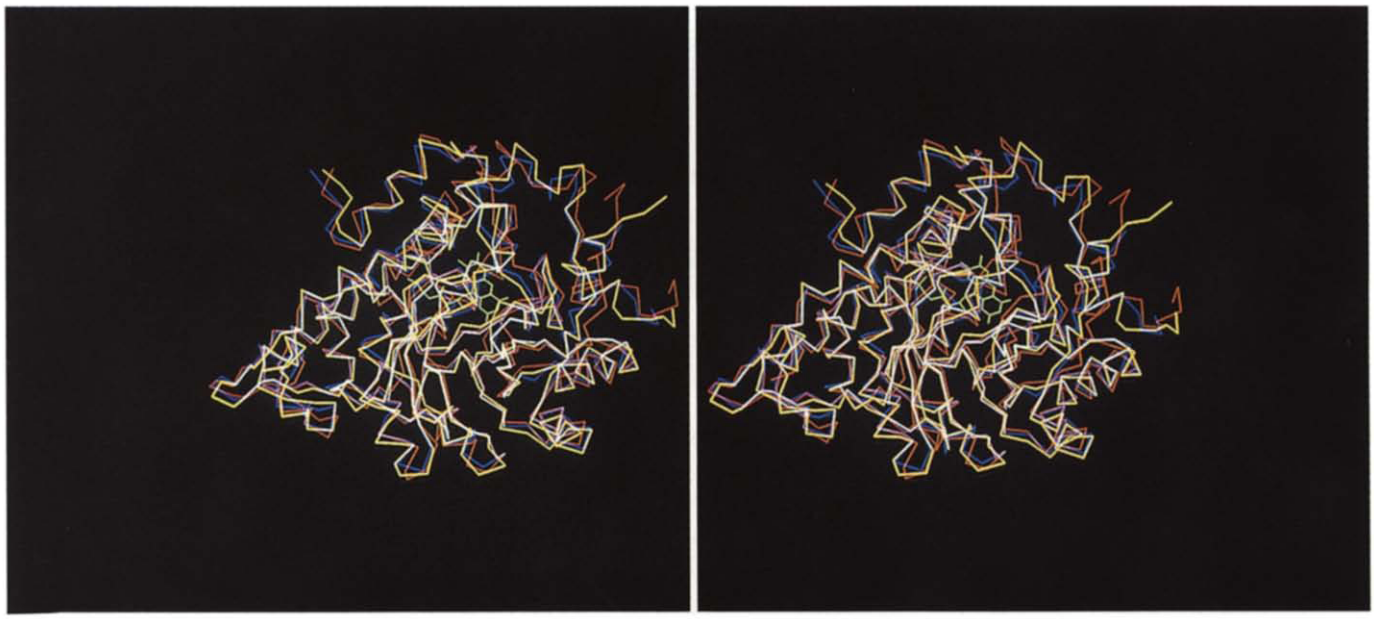

Fig. 2. Stereo view of the superposition of the C_α-trace of holo-glycolate oxidase (blue) and the two subunits of apo-glycolate oxidase (red and yellow).

than those at the C-terminal end of the barrel. Similar results were obtained even for those α/β-barrel proteins which do not share sequence homology: loops at the amino termini of the barrel frequently belong to one of a few distinct structural loop motifs with conserved structure [29]. Loops at the opposite side of the barrel are structurally quite different, they are responsible for coenzyme and/or substrate binding and active center formation. Flavin dissociation causes disordering of these loops.

About 15 residues were found in the energetically unfavorable regions of a Ramachandran map in each subunit of apo-GOX. Both $|F_o|-|F_c|$ delete map and omit maps showed that the majority of them are well defined in electron density. Seven residues were in the forbidden region of Ramachandran plot for the native GOX, which is refined to high resolution [7]. All of them (or their neighbors) remain outside favorable regions in the apo-enzyme as well but the value of the dihedral angles may change. One can divide all residues with unusual main chain conformation into three groups. First, residues from regions which have ill-defined electron density (residues 19, 137, 200, 330). Second, residues from intersubunit contact areas (residues 13, 19, 87). Third, residues from flexible loops near the flavin binding site. There are three loops in GOX which cannot be folded without tension in the chain. These are the loop between helices B and C (residues 29–32), loop 1 between 1 strand and helix 1 (residues 78–88), and loop 6 (residues 253–264). These loops contain residues with unfavorable backbone conformation in all three subunits: in holo-enzyme and in both subunits of apo-enzyme.

In the native enzyme loop 29–32 forms type II turn with Glu-30 in the position which is usually occupied by glycine [7]. In the apo-enzyme subunits, this loop has different conformations: Glu-30 is in favorable region but neighboring residues have obtained forbidden values for the dihedral angles. As a result, the loop in subunit 2 has moved away from loop 6, but in subunit 1 these two loops have come closer.

Loop 1 is part of the FMN binding site: Thr-78 is packed very close to isoalloxazine which results in an unusual geometry. Dissociation of flavin releases this loop, Thr-78 and Ala-79 can return to the favorable region (subunit 1), but a main chain hydrogen bond between residue 78 and 81 (O–N distance is 2.9 Å) keeps Ala-79 in unfavorable conformation in subunit 2. The result is that loop 1 and 2 are closer together in space in subunit 1 than in holo-GOX but in subunit 2 they are further apart than in holo-GOX.

Loop 6, the most important in FMN binding and catalytic function, has two residues (Gln-258 and Tyr-261) in a forbidden region in holo-enzyme. This loop becomes very flexible and is poorly defined in electron density. Nevertheless, it can be seen that the conformation of this loop is very different in the two subunits of the apo-enzyme. In subunit 2, main chain 253–257 residues moved only slightly. The side chain of His-254 and Arg-257 are now in the position that was occupied by the isoalloxazine ring in holo-GOX. Arg-257 has more extended conformation than in holo-GOX and there is

a shift of the rest of this loop by 1.5–2 Å. In subunit 1, His-254 and Arg-257 moved away from the former coenzyme position and all other residues of this loop are shifted out towards solution.

FMN dissociation from glycolate oxidase results in gross changes in the protein structure. It appears as if flavin dissociation transfers the protein into a very flexible non-compact state, almost similar to a molten globule, with secondary structures as in holo-enzyme but with a variable tertiary structure from which the crystal lattice forces have picked out two of many equally possible conformations. This is an uncommon situation; when coenzyme binding does not occur in interdomain or intersubunit regions, its dissociation usually causes conformational changes of some flexible loops. Interdomain/intersubunit coenzyme binding might result in rigid body movement of whole domains [12] but not always: in transketolase, where the coenzyme is bound between different subunits, the rms distance in position of 656 superimposed C_α atom of apo- and holo-enzyme is 0.53 Å and the main difference is the conformation of two flexible loops which might be in 'open' and 'closed' conformation [14].

The conformational changes which have been observed in α/β-barrels are the result of substrate binding and are also mainly made up from movement of flexible loops (loop 6 for triosephosphate isomerase [30] and Rubisco [31]). Flavin is bound at the same position as these substrates but its dissociation seems to influence the structure of the α/β-barrels much stronger.

Similar results to what has been gained here were obtained for glutathione reductase [18]. It was shown that the apo-enzyme is generally much less rigid and that conformational changes upon FAD binding to apo-enzyme are much larger than those upon NADPH binding.

From this and earlier studies the simplistic conclusion can be drawn that the influence of coenzyme on protein structure is directly dependent on two factors: the strength of coenzyme binding and the flexibility of protein elements participating in binding. It is related to the amount of surface area that becomes exposed in the apostructure after cofactor dissociation if one compares FMN/GOX (destabilization, 800–950 Å^2), with ThDP/transketolase (2 loops flexible, 0–250 Å^2) and NADH/alcohol dehydrogenase (domain rotation, 200–450 Å^2).

Our results together with those by Schultz and Ermler [18] give a structural view of flavin influence on protein structure and confirms the importance of flavin binding, whether it is FMN or FAD, to protein structure integrity.

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