Glycerol-assisted Restorative Adjustment of Flavoenzyme Conformation Perturbed by Site-directed Mutagenesis*

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Andrei A. Raibekas and Vincent Massey‡
From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109-0606

The replacement of histidine 307 with leucine in pig kidney β-amino acid oxidase perturbs its active site conformation accompanied by dramatic losses in protein-flavin interactions and enzymatic activity. However, the negative effect of this mutation on the holoenzyme structure is essentially eliminated in the presence of glycerol, resulting in up to 50% activity recovery and greater than 16-fold increase in the flavin affinity. Further analysis revealed that glycerol assists in the rearrangement of the protein toward its holoenzyme-like conformation together with reduction in the solvent-accessible protein hydrophobic area as demonstrated by limited proteolysis and use of affinity and hydrophobic probes. A substantial decrease in the protein-flavin interactions was demonstrated at a low temperature, but this reversible process was completely blocked in the presence of 40% glycerol. We suggest that the perturbation of the β-amino acid oxidase active site is due to the nonpolar nature of the mutation whose negative impact on the holoenzyme structure can be overcome by glycerol-induced strengthening of protein internal hydrophobic interactions.

The versatile properties of the flavin prosthetic group located in the active site of flavoenzymes have been successfully used over the years to study enzymatic redox mechanisms, including flavin-mediated activation of molecular oxygen (1, 2). In addition, the spectral redox properties and the reactivity of the flavin can be greatly manipulated by its modification with various chemical groups, a feature that has been particularly useful for structure functional analysis of flavoproteins (3–8). flavin can be greatly manipulated by its modification with various chemical groups, a feature that has been particularly useful for structure functional analysis of flavoproteins (3–8).

EXPERIMENTAL PROCEDURES

Materials—β-phenylglycine, FAD, phenylmethylsulfonyl fluoride were from Sigma. Bis(5,5'-8-anilino-1-naphthalenesulfonic acid (bis-ANS) was from Molecular Probes. Pure pig kidney DAAO apoprotein was from Calzyme (San Luis Obispo, CA). Trypsin (2 × crystalized) was from Merck. (400 × 1 cm) was from Aldrich. Pre-mixed SDS-PAGE protein standards were from Sigma and Bio-Rad.

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Preparation and Analysis of Tryptic Digests of DAAO—The DAAO

1 The abbreviations used are: DAAO, β-amino acid oxidase; H307L, Hist-307 → Leu (H307L) recombinant mutant of pig kidney β-amino acid oxidase (DAAO)3 (10) as a model system. Hist-307 is one of two residues (the other is Tyr-228) undergoing affinity labeling with the β-propargly-cine-suicide substrate of pig kidney DAAO (11–13). Furthermore, the replacement of His-307 with Leu greatly reduces the protein affinity for FAD so that DAAO can be isolated only as an inactive apoprotein (14, 15). Although this is an indication that the presence of His-307 is important for protein-flavin interactions, the role of this residue remains vague (15). The recently determined crystal structure of pig kidney DAAO shows that although His-307 is located in the flavin binding domain it is not closely positioned toward FAD (16, 17).

In this work we demonstrate the recovery of flavin binding and activity of the H307L recombinant mutant of pig kidney DAAO in the presence of glycerol through the proposed glycerol-induced increase in protein internal hydrophobic interactions (9) and suggest an essential role of His-307 in maintaining the proper conformation of the DAAO flavin binding domain.

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Glycerol as Chemical Chaperone

RESULTS AND DISCUSSION

Glycerol-assisted Restoration of Catalytic Activity and Flavin Binding of H307L DAAO—H307L DAAO was purified from 40–50 g of E. coli cell paste according to the standard procedure (15) and generally yielded 5–7 mg of pure 39-kDa polypeptide as judged by SDS-PAGE. The purified protein contained no flavin prosthetic group as judged by its absorbance spectrum, which is in agreement with data reported earlier (15). Although the protein displayed no activity, up to 7% of its catalytic activity was recovered when a sample was preincubated with FAD and various concentrations of glycerol, followed by activity measurements (see Fig. 1A legend for details). It should be noted that the final concentration of glycerol in the assay mixture was always less than 1%. Fig. 1A illustrates a dramatic increase in the H307L DAAO activity with the corresponding increase in glycerol concentration (5–40%, v/v) resulting in a recovery of up to 50% activity as compared with that of the wild type enzyme. The 15-min preincubation with FAD at room temperature was found to be appropriate to yield a maximum DAAO activity for each concentration of glycerol used in the preincubation mixture (data not shown). These results suggested that glycerol could have a restorative effect on the conformation of the H307L DAAO flavin binding site, which had been perturbed by mutagenesis.

In fact, the strength of the protein-flavin interactions was dramatically increased in the presence of glycerol. As a result, the dissociation constant was exponentially changed from 133 μM down to 8 μM in the 0–20% region of glycerol concentration (Fig. 1B). The glycerol-dependent increase in the flavin binding to the protein in the presence of an excess of FAD resulted in an estimated 0.56 mol of bound FAD per mol of protein in 20% glycerol (data not shown). Therefore, the recovery in the H307L DAAO activity corresponded to the increased amount of protein-bound flavin at these experimental conditions, suggesting that while the conformation of the DAAO flavin binding site was perturbed by mutation, this perturbation tended to become negligible in the presence of glycerol and FAD. The glycerol-induced activation and flavin binding were fully reversible processes, i.e. when glycerol and the excess of FAD were removed from the sample solution by dialysis, the resulting protein contained no flavin and displayed no activity, but could be reactivated again in the presence of FAD and glycerol.

Limited Proteolysis of H307L DAAO—To obtain more information about the structural characteristics of H307L DAAO in comparison with those of the wild type enzyme, we tested susceptibility toward proteolytic attack with trypsin followed by SDS-PAGE analysis. In fact, a clear difference was observed after 1 h of proteolysis at 37°C in the absence of FAD, resulting in the appearance of an additional 5-kDa band in H307L DAAO digest but not in that of wild type apoprotein (Fig. 2A, lanes 4 and 8). When 15 min of incubation with FAD (no glycerol) was introduced prior to digestion, the resulting digest of the reconstituted apoprotein displayed a pattern characteristic of the holoenzyme (25), consisting of major 25- and 14-kDa fragments, and a minor 13-kDa peptide (Fig. 2A, lanes 5 and 9), whereas the digested mutant displayed a mixed apo- and holoenzyme pattern, including the presence of the distinguishing 5-kDa fragment (Fig. 2A, lane 2). In contrast, tryptic digestion of the mutant enzyme in the presence of FAD and 20% glycerol showed the characteristic holoenzyme pattern with the 5-kDa peptide no longer present in the digest (Fig. 2A, lane 3). In a separate experiment (Fig. 2B), the digestion of H307L DAAO was conducted at 25°C in the presence of increasing concentrations of glycerol (0, 10, and 20%) with or without FAD, clearly demonstrating the glycerol-dependent transition of H307L DAAO from the perturbed binding site toward its native-like conformation. The N-terminal sequence of the 13- and 14-kDa fragments derived from the digestion of the enzyme in its native conformation was determined as GIYNSP (see details.
increased the fluorescence intensity of bis-ANS bound to either H307L protein) has been previously demonstrated (30). The difference in protein-to-protein affinity. The curve fit analysis resulted in $K_{\text{d, app}}$ values to be at least 4-fold higher for the samples containing 20% glycerol (data not shown). Furthermore, the difference in the fluorescence intensity of bis-ANS bound to either H307L DAAO or wild type apoprotein in 20% glycerol was about 50% less than that obtained without glycerol. It must be noted that the shape and the maxima of the fluorescence emission spectra were indistinguishable in the case of probe bound to either mutant or wild type apoprotein (data not shown). Taken to-

under “Experimental Procedures”), indicating that both peptides were generated as a result of the tryptic cut at Arg-221 similar to that reported earlier for wild type holoenzyme (25). However, the 5-kDa fragment generated solely during the H307L DAAO digestion was also produced as a result of the same cut at Arg-221 based on its N-terminal sequence analysis. Subsequently, the peptide molecular weight of 4908 daltons was determined by MALDI analysis. The fragment of this particular size (theoretically the calculated value is 4907.48) can be generated only if the second cleavage site is Arg-265 based on the known DAAO primary structure (28). The data suggest that while H307L DAAO has a perturbed structure, an enhanced proteolytic susceptibility of the C-terminal part of the probe was bound to the mutant was about 40% lower than that found with the wild type apoprotein. Different results were obtained when the same experiment was conducted in the presence of 20% glycerol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the relative fluorescence corrected for dilution. Control experiments showed no difference in the fluorescence intensity (at 480 nm) between 1.2 μM bis-ANS in either 80% methanol/water (v/v) or 80% methanol/ glycerol (v/v).

FIG. 3. Limited proteolysis shows the structural difference between the H307L and wild type DAAO and glycerol-mediated transition of the mutant toward the native-like conformation. Samples were preincubated for 15 min at room temperature before the addition of trypsin. FAD was added in 10-fold excess over the protein. Panel A, tryptic digestion of protein samples was conducted for 1 h at 37 °C, and digests were analyzed by SDS-PAGE. Lane 1, undigested DAAO; lane 2, mutant + FAD; lane 3, mutant + FAD + glycerol; lane 4, mutant + FAD + glycerol; lane 5, wild type DAAO + FAD + glycerol; lane 6, wild type DAAO + FAD; lane 7, apoprotein; lane 8, apoprotein + FAD; lane 9, apoprotein + FAD + glycerol. Glycerol was added to 20% (v/v) final concentration. Panel B, tryptic digestion of H307L DAAO was conducted for 3 h at 25 °C. Lanes 1–3, protein digests in 0, 10, and 20% (v/v) glycerol, respectively; lanes 4–6, same as lanes 1–3 but preincubated with FAD.

FIG. 2. Glycerol-assisted reduction in discrepancy between H307L and wild type DAAO surface conformations as probed by bis-ANS. Panel A, 1 μM bis-ANS was titrated with either H307L DAAO (●) or wild type apoprotein (□) in 50 mM sodium pyrophosphate buffer, pH 8.5, for 10 min at 21 °C. The fluorescence emission of the protein-bound probe was measured at 480 nm with excitation at 370. Panel B, same as that described in panel A but in the presence of 20% glycerol.

DAAO or wild type apoprotein is shown in Fig. 3. The titration of 1 μM bis-ANS with the protein in the absence of glycerol resulted in an increase of the bis-ANS fluorescence, which corresponds to the amount of the protein-bound probe under these experimental conditions. The titration curves reached near saturation at 3 μM protein concentration, indicating that most of the probe was bound to the protein (Fig. 3A). The stoichiometric binding of probe (i.e. one accessible bis-ANS binding site) to either protein was estimated based on the intercept of two lines drawn from the initial and final close to linear segments of the saturation curve. The dissociation constant ($K_{\text{d}}$) values for bis-ANS binding estimated as 0.53 and 0.59 μM for H307L DAAO and wild type apoDAAO, respectively, were quite similar. However, the fluorescence intensity of the probe bound to the mutant was about 40% lower than that found with the wild type apoprotein. Different results were obtained when the same experiment was conducted in the presence of 20% (v/v) glycerol (Fig. 3B). Both titration curves failed to show saturation behavior, i.e. there was a weaker protein-to-probe affinity. The curve fit analysis resulted in $K_{\text{d, app}}$ values to be at least 4-fold higher for the samples containing 20% glycerol (data not shown). Furthermore, the difference in the fluorescence intensity of bis-ANS bound to either H307L DAAO or wild type apoprotein in 20% glycerol was about 50% less than that obtained without glycerol. It must be noted that the shape and the maxima of the fluorescence emission spectra were indistinguishable in the case of probe bound to either mutant or wild type apoprotein (data not shown). Taken to-
Together, these results clearly demonstrate the differences in conformation between mutant and wild type apoprotein, indicating a greater polarity of the microenvironment around the mutant-bound probe (31). On the other hand, they also suggest two different events occurring after both proteins were placed in 20% glycerol. First, there was a reduction in the difference between the two protein conformations. Second, the bis-ANS binding site itself became less hydrophobic with either protein, indicating reduced accessibility of some site-contributed nonpolar amino acid residues.

To determine whether bis-ANS was bound at near the DAAO flavin binding site, we conducted a “competition” experiment in the presence of bis-ANS and 8-mercapto-FAD. The latter was chosen instead of FAD due to its ability to generate a longer wavelength absorbance spectrum (maximum at 595 nm) upon binding to the DAAO apoprotein (32). The DAAO apoprotein (3 μM) preincubated with various (0–100 μM) bis-ANS concentrations was mixed with 6 μM 8-mercapto-FAD, and the flavin binding process was monitored by an increase in absorbance at 650 nm during 30 min of incubation at 18 °C. As shown in the Fig. 4, bis-ANS effectively competed for the flavin binding site of DAAO, resulting in over 60% reduction in the amount of protein-bound flavin in the presence of 14 μM bis-ANS. No binding of 8-mercapto-FAD to the protein was observed when the bis-ANS concentration in the incubation mixture was increased up to 100 μM.

Hydrophobic Interactions and Glycerol-assisted Recovery of H307L DAAO Flavin Affinity—The correlation between the glycerol-mediated reduction in the bis-ANS-accessible hydrophobic area of H307L DAAO and the increase in its flavin affinity prompted us to further investigate the possible relationship between the strength of the protein internal hydrophobic interactions and protein-flavin interactions. The H307L DAAO preincubated at 18 °C with an excess of 8-mercapto-FAD and 0–40% (v/v) glycerol was placed at 0 °C with subsequent re-incubation at 18 °C. Fig. 5 shows the effect of both glycerol and the temperature transition on the flavin binding. As anticipated, the amount of protein-bound flavin analogue at 18 °C was dependent upon glycerol concentration and reached about 0.8 mol per mol of protein in 40% glycerol, giving a higher value than that estimated in the case of FAD in 20% glycerol (see above). This is likely to be an effect of the higher glycerol concentration and/or due to the fact that 8-mercapto-FAD displays a higher affinity toward the DAAO apoprotein as compared with that of the native FAD (32). A significant decrease in the protein-flavin interaction was observed when the temperature of the sample was lowered to −0 °C. The observed effect was a reversible process, resulting in complete recovery of initial flavin binding after the temperature was raised back to 18 °C. The low temperature-induced decrease in the flavin affinity was clearly dependent on the presence of glycerol in the sample solution and completely prevented in 40% glycerol. It is known that lower temperatures can have a destabilizing effect on protein conformation and in certain cases can lead to cold denaturation, which appears to be a result of a partial disruption of the protein internal hydrophobic network caused by the hydration of nonpolar groups (33). On the other hand, it has been previously suggested that the stabilizing and chaperone-like effects of glycerol on protein conformation in vitro are due to its preferential exclusion from the protein-solvent interface that, in turn, would favor strengthening of protein internal nonpolar interactions (9, 34–36). Hence, in the case of H307L DAAO it is reasonable that (i) the observed decrease in protein-flavin interactions was due to a conformational change resulting from the low temperature perturbation of the protein internal hydrophobic network, and (ii) this reversible process tended to become negligible with increased glycerol concentration as a result of the strengthening of protein internal hydrophobicity and was accompanied by glycerol-induced reduction in the solvent-accessible (external) protein hydrophobic area. Taken together, our data suggest the existence of a direct relationship between increase in the strength of protein internal hydrophobicity and flavin affinity and support the idea of hydrophobic force being a predominant factor in the glycerol-assisted process of rearrangement of the H307L DAAO molecule toward its holoenzyme-like conformation.

How Can Replacement of Histidine 307 with Lecine Perturb the Flavin Binding Site of α-Amino Acid Oxidase?—Since the crystal structure of pig kidney DAAO has been solved recently at 2.6 Å resolution (16), we were able to obtain additional information regarding His-307 and its vicinity as illustrated in Fig. 6. His-307 is located on the protein C-terminal β-strand (βF6), which is a part of the six-stranded β-sheet and the flavin binding domain (16). Although the minimal distance between this residue and FAD (the adenylate portion) is about 12 Å, close inspection reveals that the replacement of the polar His-307 with a nonpolar residue such as Leu could have a destabilizing effect on the proper configuration of the flavin binding domain. His-307 is surrounded by a polar Asn-180 (βF4), Arg-290 (loop) and Glu-294 (βF5) within van der Waals distance (2.9–3.3 Å). The polar nature of this particular site would imply its solvent accessibility, which is consistent with an ability of His-307 to be targeted and labeled by DAAO-reactive agents.
such as D-propargylglycine (13) and 9-azidoacridine (37). Moreover, the above mentioned Asn-180, Arg-290, Glu-294, and His-307 itself are conserved residues based on the amino acid sequence comparison of D-amino acid oxidases from various sources such as yeast, fungus, and mammals (38). On the other side, there is a nonpolar cluster, 3.7–6.8 Å away from His-307 that is represented by Phe-167, Leu-296, and Val-305. Hence, there is a nonpolar cluster, 3.7–6.8 Å away from His-307 and its vicinity.

In summary, we have demonstrated the restorative effect of glycerol on the conformation of pig kidney D-amino acid oxidase perturbed by site-directed mutation. The data reported here are consistent with recent findings, suggesting that (i) glycerol may act as a chemical chaperone in various protein model systems (9, 39, 40), and (ii) the hydrophobic force is a key factor in the glycerol-assisted process of adjustment of the protein molecule toward its native conformation (9). We also have attributed the perturbation of the flavin binding site of H307L DAAO to the nonpolar nature of the mutation whose negative impact on the protein structure can be overcome by glycerol-induced strengthening of protein internal hydrophobic interactions.

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FIG. 6. Fragment of DAAO structure (16) illustrating the position of His-307 and its vicinity. The structure was drawn with the program INSIGHT II using the PDB coordinate file 1KIF. Loops and β-strands are shown, and α-helices are omitted for clarity. The loop described in the text is indicated by an arrow.