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Glycerol-induced development of catalytically active conformation of *Crotalus adamanteus* L-amino acid oxidase *in vitro*

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ABSTRACT **The reconstitutable apoprotein of *Crotalus adamanteus* L-amino acid oxidase was prepared using hydrophobic interaction chromatography. After reconstitution with flavin adenine dinucleotide, the resulting protein was inactive, with a perturbed conformation of the flavin binding site. Subsequently, a series of cosolvent-dependent compact intermediates was identified. The nearly complete activation of the reconstituted apoprotein and the restoration of its native flavin binding site was achieved in the presence of 50% glycerol. We provide evidence that in addition to a merely stabilizing effect of glycerol on native proteins, glycerol can also have a restorative effect on their compact equilibrium intermediates, and we suggest the hydrophobic effect as a dominating force in this *in vitro*-assisted restorative process.**

The reversible conversion of many flavoenzymes to their corresponding apoproteins can be achieved under relatively mild conditions either by the perturbation of the protein structure in low pH solution, containing high concentration of salt, or by the addition of denaturating agents such as urea or guanidinium hydrochloride (1). This approach has been successfully employed to study flavoprotein active site environment and/or the mechanism of catalysis by using various flavin analogs as spectral or chemically reactive affinity probes (2–5). However, in some cases no viable apoprotein can be prepared from the corresponding flavoenzyme due to irreversible changes in the protein structure that result in the lack of either ligand binding and/or protein solubility. In addition, the yield and stability of the apoprotein depends largely on the method of its preparation. In fact, the treatment of porcine lipoamide dehydrogenase with 5 M guanidinium hydrochloride results in unstable and poorly reconstitutable apoprotein, while the combination of low pH–high salt concentration and hydrophobic interaction chromatography leads to a high yield of its fully reconstitutable form (3, 6, 7). Still, the strategy for the development of a suitable method of apoprotein preparation from a particular flavoenzyme remains largely empirical and is simply based on screening of the various known procedures. It is conceivable that the problem of obtaining reconstitutable apoprotein is directly related to other basic problems such as the control of protein stability *in vitro* by change in its microenvironment (denaturants, stabilizers, pH, etc.) (8–11) and the existence of intermediate states of protein during unfolding–refolding processes (12, 13).

The subject of the present study was *Crotalus adamanteus* L-amino acid oxidase (LAAO). LAAO is a dimeric glycoprotein containing two molecules of noncovalently bound flavin adenine dinucleotide (FAD) per protein molecule and catalyzes oxidative deamination of various, but predominantly hydrophobic, L-amino acids (14). Our particular interest was raised by the fact that this enzyme has a highly sensitive active site, such that it undergoes reversible pH- or temperature-dependent inactivation accompanied by structural changes in flavin binding site but, at the same time, retains its overall secondary structure (15–19). The high sensitivity of LAAO to its microenvironment is one of the likely explanations for the previous failure to prepare reconstitutable apoprotein and, thus, to perform intensive structure functional studies of this enzyme by using flavin analogs.

We were able to obtain reconstitutable apoprotein of LAAO and to identify a series of cosolvent-dependent equilibrium intermediates on its *in vitro* pathway leading to the formation of the holoenzyme. We also found that glycerol as a cosolvent plays a special role in this process by induction of rearrangements in the protein structure, which in turn leads to the development of its native conformation.

MATERIALS AND METHODS

Chromatography and Electrophoresis. Analytical gel filtration studies were performed at room temperature using a Pharmacia FPLC system equipped with a Superose 12 column. A 0.1 M Tris·HCl buffer, pH 7.5, containing 0.15 M NaCl was the mobile phase. Glucose oxidase (160 kDa), threonine dehydrogenase (148 kDa), lipoamide dehydrogenase (100 kDa), albumin (66 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (12 kDa) were used as standards. SDS/PAGE was performed according to Laemmli (20) using a mini-Protean II system and premixed (broad range) protein standards (all from Bio-Rad).

Spectroscopy. Absorption spectra were recorded using a Cary 3 spectrophotometer. The concentration of LAAO was estimated based on its extinction coefficient per enzyme-bound flavin at 462 nm (ε_{462} = 10650 $M^{-1}\cdot cm^{-1}$) in 0.1 M Tris·HCl, pH 7.5. The extinction coefficient was calculated based on free flavin absorbance after the protein was precipitated with 5% trichloroacetic acid (TCA) using an extinction coefficient of free FAD in 5% TCA (ε_{450} = 10340 $M^{-1}\cdot cm^{-1}$). The latter was calculated based on the reported value at pH 7.0 (ε_{450} = 11,300 $M^{-1}\cdot cm^{-1}$) (21) and difference in extinction of FAD at neutral and acid pH.

Fluorescence measurements were conducted at 25°C using a spectrofluorimeter built at the University of Michigan. Pure FAD was used as a standard.

Circular dichroism (CD) spectra were recorded with a JASCO J-710 spectropolarimeter at 12°C using a 1 cm path length cell. The maximum of absorbance of all measured samples was <1 for either visible (VIS) or far-ultraviolet (UV) spectral regions.

Purification of LAAO. The enzyme was purified from 1 g of dry venom (Biotoxins, St. Cloud, FL) of the southeastern diamondback rattlesnake *Crotalus adamanteus* as described by Wellner and Meister (22) with few modifications. (*i*) Calcium phosphate gel was replaced with hydroxyapatite (Bio-Rad), and (*ii*) in the final step, dialysis and protein crystallization were replaced with gel filtration chromatography on a

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Abbreviations: FAD, flavin adenine dinucleotide: LAAO, L-amino acid oxidase; CD, circular dichroism; VIS, visible; Int, intermediate.
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Sephacryl S-200-HR (Sigma) column (16 × 800 mm). A 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl was used as a mobile phase (storage buffer). The purified enzyme was stored at 0°C.

Preparation of Apoprotein of LAAO and Reconstitution with FAD. The apoprotein of LAAO was prepared at 4°C by an adaptation of the described method utilizing hydrophobic interaction chromatography (7, 23). Usually, 1.5 ml LAAO storage solution (1.5 mg of protein) was mixed with 1.5 ml of buffer AA (0.1 M Tris·HCl buffer, pH 7.5, containing 0.2 M KCl, 3 M ammonium sulfate, and 1 mM EDTA) and then loaded on a small (0.8–1.0 ml) phenyl Sepharose (Pharmacia) column equilibrated with buffer A (50 mM Tris·HCl buffer, pH 7.5, containing 0.1 M KCl, 0.5 mM EDTA, and 1.5 M ammonium sulfate). The appearance of a prominent yellow band at the top of the column was an indicator of successful LAAO binding to the hydrophobic matrix. After rinsing the loaded column with at least 5 ml of buffer A, the flavin was released and eluted with 10 ml of buffer B (0.1 M phosphate buffer, pH 3.5, saturated with potassium bromide and containing 1.5 M ammonium sulfate and 0.5 mM EDTA), while the apoprotein remained bound to the column. In the following reconstitution step, 5 ml of buffer C (0.1 M Tricine buffer, pH 8.5, containing 0.2 mM FAD, 0.5 mM EDTA, 0.1 M KCl, and 1.5 M ammonium sulfate) was applied, and the column was left for 1 hr at 4°C in the same buffer and subsequently rinsed with 5 ml buffer C without FAD and 10 ml of buffer A. Finally, FAD-reconstituted protein was eluted with buffer D (50 mM Tris·HCl buffer, pH 7.5, containing 0.1 M KCl, 0.5 mM EDTA, and 50% ethylene glycol) in 2–3 ml volume.

Glycerol-Induced Activation of Reconstituted Apoprotein. Unless otherwise specified, the reconstituted apoprotein in buffer D (1–2 ml) was dialyzed overnight at 4°C against 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl and 20% glycerol. After dialysis, the sample was placed in 50% glycerol by mixing with an equal volume of 80% glycerol (vol/vol) and then incubated for a total of 3 hr at 25°C. During this time, 0.01 ml aliquots of the incubation mixture were withdrawn every 15–20 min and immediately assayed for enzymatic activity. After the activation process was complete, the sample was dialyzed overnight at 4°C against 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl (storage solution) and stored at 0°C. Slide-A-Lyzer cassettes (Pierce) were used for dialysis.

Assays. The specific oxidase activity of enzyme with L-leucine as a substrate was measured spectrophotometrically at pH 7.6 and 25°C using a coupled *o*-dianisidine-peroxidase assay (24). Protein concentration was determined using a BCA protein assay kit (Pierce) or based on the flavin absorbance (see above).

RESULTS AND DISCUSSION

Properties of Purified *C. adamanteus* LAAO. LAAO was purified from 1 g of dry venom according to the procedure described under *Materials and Methods*, and generally yielded 10–12 mg of pure protein with a specific activity of 9–10 units/mg. The resulting enzyme displayed the characteristic flavin absorbance spectrum (Fig. 1, curve 1) and a A_{280}/A_{462} absorbance ratio of 10. The gel filtration on Sephacryl S-200-HR column introduced as a final step resulted in the effective separation of enzyme from lower molecular weight impurities. The LAAO was pure as judged by SDS/PAGE and corresponded to a 68 kDa polypeptide band (denaturating conditions). The molecular weight estimated by gel filtration at native conditions on a Superose 12 column was about 120 kDa (data not shown). Therefore, the LAAO is likely to be a 130–140 kDa dimeric protein as previously suggested based on ultracentrifugation studies (25). The protein molecule contains two molecules of noncovalently bound FAD (one FAD per 68 kDa monomer) as judged by acid-induced flavin dissociation (5% TCA) and determination of protein (BCA assay) and flavin (ε_{462} = 10650 $M^{-1}\cdot cm^{-1}$) content, which is in a agreement with the value estimated by Wellner and Meister (22). The enzyme retained its activity when it was stored on ice (0°C) for two weeks in 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl.

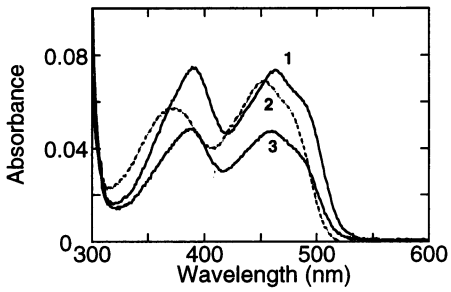

FIG. 1. Visible absorbance spectra of LAAO and its intermediates recorded at 12°C. Curve 1 shows the spectrum of the holoenzyme (7 μM) in 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl (storage buffer). Curves 2 and 3 represent the spectra of Int1 (6 μM) and Int3 (4.5 μM) in storage buffer containing 50% ethylene glycol and 20% glycerol, respectively.

Hydrophobic Properties of Apoprotein of LAAO and Its FAD-Reconstituted Form. The preparation of apoprotein of LAAO and its reconstitution with FAD on a phenyl Sepharose column are described under *Materials and Methods.* The enzyme was bound to the column at high (1.5 M) concentration of ammonium sulfate. The flavin was subsequently released by lowering the buffer pH to 3.5 in the presence of potassium bromide and then rebound at pH 8.5. The reconstitution at higher pH was essential and caused an approximately 2-fold increase in yield of the reconstituted protein compared with that at pH 7.5. The final elution step was performed with 50% ethylene glycol at pH 7.5. The eluted protein contained a stoichiometric amount of FAD and the protein yield was usually within 65–70% of the starting material.

The difference in the surface polarity of holoenzyme, apoprotein, and its reconstituted form determined the strength of their hydrophobic interactions with phenyl Sepharose, as judged by their selective elution from the column with water or 50% ethylene glycol as a stronger eluent (7, 23, 26) (Table 1). The apoprotein of LAAO was not eluted by either water or 50% ethylene glycol, while reconstitution with FAD caused a decrease in protein interaction with the hydrophobic matrix and therefore its elution with 50% ethylene glycol, but not with water. Among all three forms bound to the phenyl Sepharose, the holoenzyme displayed the lowest exposure of its nonpolar residues such that it can be eluted with either water or 50% ethylene glycol. Thus, the difference in hydrophobic properties clearly places the reconstituted protein between its apoprotein and holoenzyme.

Properties of Equilibrium Intermediates of LAAO. After elution from the column with 0.1 M Tris·HCl buffer, pH 7.5,

Table 1. Elution of different forms of LAAO from phenyl Sepharose column

Sample*	Eluent	
	H_2O	50% ethylene glycol/H_2O
Holoenzyme	+++	+++
Apoprotein	–	–
Apoprotein + FAD	–	+++
Apoprotein + FAD + activation†	+++	+++

*Prepared as described in *Materials and Methods.*
†Applied on the column at the same conditions as for holoenzyme.
+++, Elution; –, no elution.

containing 0.1M KCl and 50% ethylene glycol, the reconstituted protein displayed no activity. The spectral properties of this form, designated as intermediate 1 (Int1), were dramatically different compared with the native enzyme, as demonstrated by (*i*) perturbation in the absorbance spectrum, which resulted in a change of its shape accompanied by 10 and 19 nm shifts in maxima toward shorter wavelength, approaching the spectrum of free FAD (Fig. 1, curve 2; Table 2); and (*ii*) a 24-fold increase in flavin fluorescence up to 17% of the intensity of free FAD (Table 3) and an absence of LAAO-specific signal in the VIS (flavin) region of CD spectrum similar to that of free FAD (Fig. 3*A*, curve 2; Table 3) (19, 27). These results showed that, unlike the holoenzyme, the active site of Int1 displayed an "open" (less packed) conformation, resulting in weak interactions between the flavin ring and the polypeptide, additional exposure of hydrophobic surfaces (Table 1), and lack of catalytic activity.

Int2 was obtained by dialysis of Int1 (in 50% ethylene glycol) against 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl. Int3 was prepared in the same way except for the presence of 20% glycerol in the dialysis buffer. Although both intermediates displayed no catalytic activity, the spectral properties revealed a largely restored structure within their flavin binding sites, as demonstrated by reshape of absorbance spectra with new maxima at 389 and 460 nm similar to those of the native enzyme (Fig. 1, curve 3; Table 2). The restorative changes in absorbance were accompanied by the quenching of flavin fluorescence of intermediates and revealed 7-fold (Int2) and 14-fold (Int3) decreases in intensity of fluorescence compared with that of Int1 (Table 3). Unlike Int1, the VIS CD spectra of Int2 and Int3 were similar to the CD spectrum of holoenzyme, as demonstrated by the appearance of a pronounced positive peak at 388 nm and a broad positive shoulder in the 420–550 nm region (Fig. 3*A*). The molar CD value at 388 nm decreased in the order holoenzyme, Int3, and Int2, (Table 3), reflecting a difference in the mode of protein–flavin interactions. A similar decrease in CD signal was also demonstrated for inactivated LAAO correlating with the perturbation in its flavin absorbance spectrum (19).

When either Int2 or Int3 was dialyzed against 50% ethylene glycol (buffer D), the resulting absorbance spectra resembled that of Int1. In contrast, the holoenzyme displayed no changes in either specific activity or absorbance spectrum after dialysis vs. buffer D (Table 2). The data suggest that unlike holoenzyme, the structure of the flavin binding site of intermediates has become rather flexible and disordered and, therefore, can be further perturbed by ethylene glycol molecules bound to additionally exposed hydrophobic surfaces. This in turn can explain why the presence of ethylene glycol led to an open conformation of the flavin binding site of intermediates, but had no such effect on the holoenzyme (see *Discussion* for role of ethylene glycol).

Table 2. Absorbance characteristics of LAAO intermediates*

	Absorbance maxima, nm	
FAD	375	450†
	373	450
Int1	371	452
Int2	389	460
Int2 in 50% ethylene glycol	371	452
Int3	389	460
Int3 in 50% ethylene glycol	372	452
Int4	390	461
Int4 (dialyzed, no glycerol)§	390	462
LAAO§	390	462

*All spectra were recorded at 12°C. Samples were in their corresponding buffers, pH 7.5, as described in text unless otherwise specified.
†In storage buffer (see *Materials and Methods* for details) with or without 50% glycerol.
‡In storage buffer with 50% ethylene glycol.
§No spectral changes were observed after sample was placed in the same buffer containing either 50% ethylene glycol or 50% glycerol.

Table 3. Flavin CD and fluorescence properties of LAAO and its intermediates

	Fluorescence (%) at 525 nm*	CD signal ($\Delta\varepsilon$) at 388 nm†
FAD‡	100.0	0.2
Int1	17.0	0.1
Int2	2.5	2.5
Int3	1.2	3.7
LAAO	0.7	4.5

*Exitation at 450 nm with absorbance less than 0.1 to avoid inner filter effect. T = 25°C.
†Measured at 12°C.
‡No perturbation of signal was observed for FAD in all sample solutions.

The fully active form of the reconstituted apoprotein designated as Int4 was obtained by placing Int3 in 50% glycerol and incubating at 25°C as described in *Materials and Methods*. The activation process was accomplished in about 3 hr ($t_{1/2}$ = 45 min) and revealed a nearly complete restoration (97%) of protein specific activity (Fig. 2*A*, curve 1) accompanied by a 1 nm shift in flavin absorbance (Table 2). In contrast, the incubation of Int3 (in 20% glycerol) for 20 hr at 25°C caused only about 1% recovery of activity (data not shown). Finally, when Int4 was dialyzed against glycerol-free buffer, the resulting protein displayed properties of the holoenzyme, including surface polarity (Table 1), specific activity, absorbance spectra, and particularly, the lack of perturbation of its flavin spectrum in the presence of ethylene glycol. The removal of glycerol from the protein solution caused an additional 1 nm shift in Int4 absorbance maximum, so its absorbance spectrum became identical to that of holoenzyme (Fig. 1, curve 1; Table 2). However, the absorbance maxima of holoenzyme were

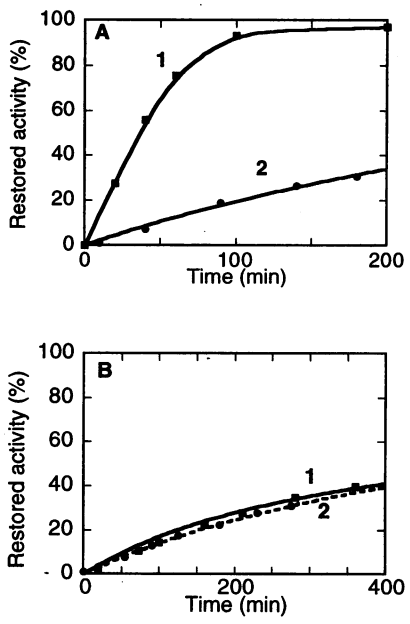

FIG. 2. Time course of activation of LAAO intermediates. (*A*) Int3 (1 mg/ml) was placed in 50% glycerol and assayed for activity as described in *Materials and Methods*. Curves 1 and 2 show the course of activation at 25°C and 18°C, respectively. (*B*) Curve 1 shows the activation profile of Int2 (1 mg/ml) after it was mixed with 100% glycerol (1:1) followed by incubation at 25°C. Curve 2 represents the activation course of Int1 (0.6 mg/ml) at 25°C predialyzed overnight at 4°C against 0.1 M Tris·HCl buffer, pH 7.5, containing 0.1 M KCl and 50% glycerol.

independent of the presence of the 50% glycerol, suggesting that the shift in absorbance may be a result of the final conformational change leading to the production of holoenzyme. The formation of Int4 was a temperature-dependent process. A slower activation was observed at 18°C and revealed only a 31% recovery of specific activity after 3 hr of incubation (Fig. 2*A*, curve 2), while 9 hr of incubation at 0°C did not show any activity restoration.

As seen from our spectral studies, Int3 exhibited great similarity to the holoenzyme, except that it lacked activity and displayed an "open" or "closed" conformation of its flavin binding site, depending on the presence of ethylene glycol. It seems clear that the restoration of the active site of Int3 during the activation process (Int3 → Int4) drastically affected its flavin binding site by locking it up in the native (closed) conformation. These data suggest that the native form of LAAO was developed through glycerol-induced structural rearrangements falling into a kinetic trap.

To obtain more information about the LAAO restoration pathway, we attempted a direct activation of Int2. No activity was detected when the sample was incubated for 20 hr at 25°C. However, when it was placed in 50% glycerol (1:1 dilution in 100% glycerol), a slow increase in activity was observed (Fig. 2*B*, curve 1) with only a 52% recovery after 30 hr of incubation at 25°C. Int1 displayed a similar activation profile after it was dialyzed against the same 50% glycerol buffer as Int2 and incubated at 25°C for 6 hr (Fig. 2*B*, curve 2). The results show that the equilibrium Int2 is not strictly required for the restoration pathway, which does not exclude its possible role as a transient intermediate. On the contrary, the formation of Int3 is an important step providing an effective restoration of native protein conformation *in vitro*.

All intermediates showed well-preserved secondary structure according to their far-UV CD spectra (Fig. 3*B*), except the overall ellipticity of Int1 decreased by ≈25%. This was perhaps due to the effect of ethylene glycol, since no perturbation was observed when Int1 was placed in ethylene glycol-free solution. The 180–200 nm area of spectra was not taken into consideration due to the high (>1) sample absorbance. In addition, the gel filtration chromatography of Int2, Int3, and Int4 on a Superose 12 column resulted in elution profiles indistinguishable from that of holoenzyme (data not shown), thereby confirming the compactness of intermediates. No protein precipitation was observed for any of the intermediates while conducting the experiments or during storage at 0°C.

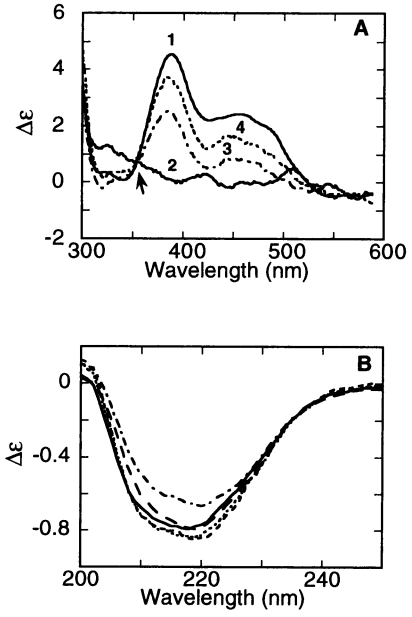

FIG. 3. CD spectra of LAAO and its intermediates recorded at 12°C. (*A*) VIS CD spectra of LAAO (curve 1), Int1 (curve 2), Int2 (curve 3), and Int3 (curve 4) in their corresponding buffers, pH 7.5, prepared as described in the text. The isosbestic point (354 nm) is indicated by an arrow. (*B*) Far-UV CD spectra of Int1 (-·-), Int2 (···), Int3 (– – –), Int4 (— —), and LAAO (——) in their corresponding buffers, pH 7.5, except for the Int4, which was in storage buffer.

Fig. 4 summarizes results obtained in this work, illustrating a series of interrelated events observed during the preparation of the apoprotein of LAAO, its reconstitution with FAD and finally, the formation of the holoenzyme. The first two steps refer to the release and rebinding of the flavin prosthetic group accompanied by corresponding changes in the properties of column-bound protein, i.e., an overall reduction in its surface apolarity. The next steps (steps 3, 4A, and 4B) cover the formation of the first three compact intermediates demonstrating reversible changes in the structure of their flavin binding sites. Step 5 illustrates the process of restoration of the protein active site and simultaneous trapping of the flavin binding site in the closed conformation, while the final step, step 6, demonstrates the release of glycerol and formation of the "relaxed" (see *Discussion* below on glycerol effect) holoenzyme. As depicted in Fig. 4, our data suggest two pathways leading to the formation of the holoenzyme. However, they do not exclude the existence of a combined single pathway:

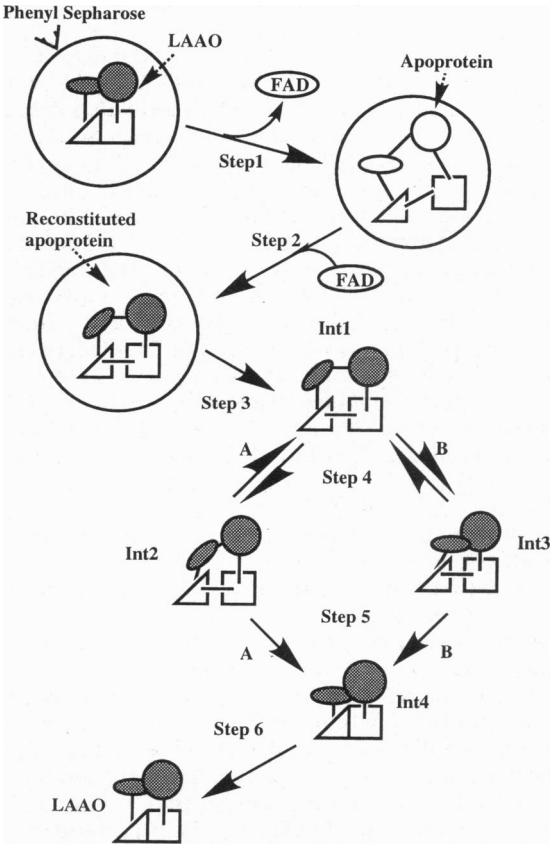

FIG. 4. Schematic presentation of proposed pathways leading to the glycerol-induced formation of the native LAAO from the corresponding apoprotein (see *Results* and *Discussion* for details). Phenyl Sepharose-bound protein is shown in enclosed circles. Shaded symbols represent protein bound to FAD in its native and intermediate states. The absence of FAD is shown by open rounds. Int1 results from elution with buffer containing 50% ethylene glycol. Int2 and Int3 represent the different protein conformations after replacing ethylene glycol with buffer containing no and 20% glycerol, respectively. Int4, with regain of catalytic activity, is obtained by activation at 25°C in 50% glycerol. Removal of the glycerol in step 6 returns the enzyme to its relaxed native conformation.

Int1→Int2→Int3→Int4. For instance, Int2 and Int3 could be transient intermediates in steps 4B and 5A, respectively. Future studies will be needed to clarify this issue.

The existence of compact intermediate states in protein folding is now a recognized fact supported by a growing amount of recent publications (12, 13). Stable compact intermediates have been observed under mild denaturating conditions, for example in low pH solutions containing moderate concentration of salt (A-states) (28–30). Also, the removal of a prosthetic group can itself destabilize the protein structure leading to the formation of an inactive reversible intermediate. It seems that conditions required for obtaining either reconstitutable apoproteins from corresponding flavoenzymes or A-states of proteins are quite similar. Nevertheless, flavoenzymes generally represent a more complicated case since additional structural rearrangements are required to restore the proper conformation of the active site (substrate and flavin binding sites) upon reconstitution of the apoprotein with the flavin prosthetic group. All these aspects were particularly visible in the case of LAAO and described in the present study. Unlike many other flavoenzymes, the rebinding of flavin to the apoprotein of LAAO does not restore the proper conformation of its active site. At this point, an inactive form of the enzyme appears to be thermodynamically favorable and represented by cosolvent-dependent equilibrium intermediates (Int1, Int2, and Int3). Then, the addition of glycerol shifts the equilibrium from the inactive toward the active conformation in a temperature-dependent manner.

Why does glycerol have a pronounced restorative effect on enzyme structure and ethylene glycol does not? The problem of microenvironment-controlled stabilization–destabilization of proteins was poorly understood until recently, when Timasheff and colleagues (10, 31) proposed a universal concept based on the preferential exclusion of stabilizers from protein–solvent interface and reversibly, the preferential binding of denaturant to the protein surface. Glycerol has been known over decades as one of the most frequently used stabilizers of protein native structure (32–35). In fact, glycerol was found to be preferentially excluded in a nonspecific manner from the domain of the protein (solvophobic effect). This in turn creates a thermodynamic situation such as more free energy being required for protein unfolding than in water. Also, it has been suggested that the stabilization effect is a result of a certain balance between the repulsion of glycerol from hydrophobic surfaces of protein and its simultaneous interaction with protein polar regions (36, 37).

One of the most prominent characteristics of compact intermediates compared with native proteins is a partially disrupted network of internal hydrophobic interactions leading to a corresponding increase in the amount of solvent-exposed nonpolar surfaces (12). Therefore, placing of a protein intermediate in glycerol, in principle, may initiate restoration of the internal protein hydrophobic core through the above mentioned repulsion mechanism. Our results completely support this idea, i.e. the presence of high glycerol concentration induces repacking of LAAO Int2 and Int3 and drives them into a "conformational trap" designated for correctly folded protein (Fig. 4, step 5). This allowed us to conclude that in addition to a merely stabilizing effect of glycerol on native proteins, it can also have a restorative effect on their partially unfolded intermediates. The described restorative feature of glycerol potentially could find very useful and broad application, because it seems to affect proteins in a nonspecific manner (see above), so it could be used together with molecular chaperones (or, in some cases, even as an alternative) to assist correct folding of various proteins *in vitro*. For example, recently we found (unpublished results) that the conformation of flavin binding site of D-amino acid oxidase disrupted by site-directed mutagenesis can be restored in the presence of glycerol. Moreover, according to Sato *et al.* (38), the relatively low (10%) concentration of glycerol can mediate correct folding of mutated (ΔF508) cystic fibrosis transmembrane conductance regulator in mammalian cells at higher (37°C) temperature. Glycerol may also affect the structure of native proteins through the same repulsion mechanism assuming the protein has solvent-exposed nonpolar residues together with flexible regions, which often are distributed within its active site. In fact, glycerol-dependent changes in tryptophan phosphorescence at various temperatures have been observed in the case of four different proteins including *Escherichia coli* alkaline phosphatase and liver alcohol dehydrogenase (39), whereas Pourplanche and coworkers (40) have shown structural changes in the active site of soybean lipoxygenase induced by glycerol or sugars. In addition, it has been recently demonstrated that the volume of the protein interior is decreased in the presence of glycerol (41).

Unlike glycerol, the presence of ethylene glycol favors the unpacked conformation of the Int1 flavin binding site. This is in good agreement with the previously demonstrated temperature-dependent destabilizing effect of ethylene glycol on protein structure and the suggested nonpolar nature of protein-ethylene glycol interactions (42–44). In hydrophobic interaction chromatography, which appears to be a useful tool to monitor changes in protein surface polarity (ref. 45; this work), ethylene glycol acts as a strong eluent and thus, effectively competes for protein and/or matrix nonpolar surfaces (7, 23, 26). Moreover, the increase in temperature was found to be an important factor in achieving the successful elution of chymotrypsin, chymotrypsinogen A and apolipoamide dehydrogenase from the hydrophobic matrix with ethylene glycol (ref. 46; unpublished results), which in turn would imply an increase of the strength of nonpolar interactions (47, 48). Thus, it is conceivable that the observed perturbation of the flavin binding site of Int1 is a result of a local disordering of the native structure of LAAO, which in turn leads to an expansion of hydrophobic surfaces preferentially interacting with ethylene glycol rather than with either water and/or glycerol molecules (Int2 and -3). Then, the glycerol-induced collapse of the hydrophobic core followed by the restoration of its native structure largely reduces surface apolarity, so that the flavin binding site can no longer be perturbed by ethylene glycol.

Although the question of dominating forces in protein folding remains largely controversial, the hydrophobic effect is perhaps a key factor involved in the restriction of the conformational space of protein and thus, the selection of a small number of compact intermediates on the pathway leading to an accumulation of the final product, i.e., native structure (12, 49–54). Indeed, the results obtained in this work suggest the hydrophobic effect as a dominating force in an observed *in vitro* process leading to the glycerol-induced development of the native state of *C. adamanteus* LAAO from its compact equilibrium intermediates.

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