with exogenous cytochrome *c* is somewhat greater if the ATP is added after the reaction is initiated. Whatever the change resulting from its presence, it is not specific for ATP, since inorganic pyrophosphate shares these effects.

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Cryoenzymology and Spectrophotometry of Pea Seedling Diamine Oxidase†

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ABSTRACT: Diamine oxidase follows bi-ter ping-pong kinetics, with an intermediate, "reduced" free-enzyme form being generated after the anaerobic conversion of amine to aldehyde. Visible spectra of diamine oxidase reacting at subzero temperatures provide evidence that this intermediate enzyme form is obtained via several other intermediates and that the environment of the Cu(II) changes dramatically during the course of the reaction [even though it is not reduced to Cu(I) during the catalytic cycle]. The spectrum of this form of diamine oxidase, which is obtained 0.5–2 h after the addition of amine at −5 to −15 °C, is independent of substrate, is identical with that obtained by anaerobic addition of substrate at room temperature, and provides evidence for a direct interaction of Cu(II) with the organic cofactor of the enzyme. This interaction is apparently charge transfer in nature. Upon removal of Cu(II) from the native enzyme, one obtains spectral evidence that the organic cofactor is still present. However, removal of the Cu(II) from the reduced (intermediate) enzyme form yields a featureless enzyme spectrum and a Cu(II)–chelate complex which contains a new ligand, which is presumably the second prosthetic group.

Diamine oxidase [DAO;[1] diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6] catalyzes the oxidative deamination of a variety of amines according to the general reaction

$$R\text{–}CH_2NH_2 + O_2 \rightarrow R\text{–}CHO + H_2O_2 + NH_3$$

where R equals $NH_2(CH_2)_3$– (putrescine), C_6H_5– (benzylamine), or 4-$(CH_3)_2NCH_2C_6H_4$– [*p*-[(dimethylamino)methyl]benzylamine]. The work described in this paper was performed by using pea seedling DAO; previous work has dealt with the porcine kidney enzyme (Kluetz & Schmidt, 1977a,b), which also attacks histamine but does not oxidize benzylamine.

DAO has been shown to follow bi-ter (uni uni uni bi) ping-pong kinetics [Bardsley et al., 1973; Nylēn & Szybek, 1974; notation and schematics of Cleland (1963)], in which 1 equiv of amine is converted to the corresponding aldehyde in an anaerobic stage, leaving the enzyme in an intermediate, "reduced" form (designated E′); the E′ form of DAO is subsequently reconverted to the native state upon reaction with O_2, eliminating H_2O_2 and NH_3:

amine	aldehyde	O_2	H_2O_2	NH_3
↓	↑	↓	↑	↑
E		E′		E

DAO requires as cofactors Cu(II) and an as yet unidentified organic moiety, and we have been particularly interested in

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[1] Abbreviations used: DAO, diamine oxidase; DAB, *p*-[(dimethylamino)methyl]benzylamine; PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Me_2SO, dimethyl sulfoxide; DDC, diethyldithiocarbamate.

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the interaction of these groups both with the enzyme and with each other. We have previously used magnetic resonance methods to study the nature of the Cu(II) site in both the porcine and pea systems and to study the binding of Cu(II) to apoDAO using the latter enzyme (M. D. Kluetz and K. Adamsons, unpublished results). In hog kidney DAO, the Cu(II) is at least 8 or 9 Å from the oxidation site. Similarly, in the pea seedling enzyme, the product analogue dimethylamine binds over 8 Å from the Cu(II), but, as we will show in this work, the metal ion does interact with the organic cofactor. Cu(II) is not reduced to Cu(I) during the course of reaction in either system.

It has been suggested that pyridoxal-P (PLP) is the organic cofactor of DAO, there being more evidence in support of this conclusion in the case of porcine DAO (Mondovì et al., 1967; Werle & von Pechmann, 1949). The amine oxidases are unique in their requirement for both Cu(II) and this type of organic group. The optical spectrum of hog kidney DAO reveals a strong band at 405 nm, which makes the enzyme visibly yellow; this type of absorption band is reminiscent of those found for PLP Schiff's bases. Nonetheless, other workers have argued that PLP is not the cofactor of this enzyme (Inamasu et al., 1974). In striking comparison to the hog kidney enzyme, concentrated solutions of pea seedling DAO are bright pink rather than yellow (vide infra). Despite the similar substrate specificities, physical properties, and reaction mechanisms, the two enzymes appear to be quite different in terms of their prosthetic groups.

We have attempted to further elucidate the nature of the cofactor interactions in DAO using optical spectroscopy of modified and reacting enzyme. As we will show, one obtains significantly more spectral information by observing the intermediate, E′ state. The enzyme can be "trapped" in this form by the anaerobic addition of substrate. Hill & Mann (1964) had previously observed a pink to yellow transition in pea seedling DAO upon anaerobic addition of amine, although their interpretation of the nature of the yellow species appears to be incorrect.

Although DAO can be trapped in its intermediate state by the above method, one finds that the changes observed unfortunately occur in too short a time to be of maximum value. However, by the application of cryoenzymologic techniques, one can slow this transition sufficiently to spectrally observe the formation of intermediate states using conventional repetitive-scanning spectrophotometry. If one runs an enzyme-catalyzed reaction at subzero temperatures (in a partially organic solvent system), one can slow the reaction tremendously. Under such conditions, one observes the reaction under nonturnover conditions, and various reaction intermediates can be temporally "isolated" and observed. It is advantageous to have informative spectral bands in the visible region, as in the case of DAO; in the absence of such chromophores, one needs to observe the main protein band itself, the appearance of an absorbing product, or the disappearance of an absorbing reactant. [For reviews of several studies of this nature, see Fink (1977), Makinen & Fink (1977), and Douzou (1974, 1977).]

Materials and Methods

Enzyme Preparation. Electrophoretically pure pea seedling DAO was obtained by a procedure developed in our laboratory. The specific activity with putrescine is 40–50 units/mg (37 °C). Concentrations of pure DAO are calculated from the absorbance at 280 nm with $A_{280}^{1\%} = 13.8$ cm^{-1}. The enzyme is freed of all but active-site Cu(II) by dialysis against EDTA, followed by exhaustive dialysis against P_i buffer.

Optical Spectra. Visible spectra were obtained on a Beckman ACTA MVII ultraviolet/visible/near-infrared spectrophotmeter (also equipped for repetitive scanning).

Reaction and Spectrophotometry at Subzero Temperatures. Enzyme solutions for the low-temperature reactions were prepared as follows: equal volumes of Me_2SO (Mallinckrodt) and 0.1 M P_i buffer are mixed and the "pH" (pa_H) is set to 7.0 (Radiometer Model 26 pH meter); 0.8 mL of this solution is cooled to between −10 and −15 °C, and to it is added 0.2 mL of DAO solution at 0 °C (in 0.05 M P_i; DAO concentration is 5 times that desired in the final solution); this enzyme solution is centrifuged for 30 min at 10000 rpm (Sorval RC-5B) at −15 °C; finally, the solution is transferred to a precooled spectrophotometer cell where the temperature at which the reaction is to be run is maintained with a Neslab Cryo-Cool 60 circulating low-temperature bath. The cell itself is a Hellma jacketed, 1-mL quartz cell (path length of 1 cm). Alternatively, one may use a brass block cell holder, which is cooled by a flow of refrigerant, and a standard 1-mL cell. Condensation on the cell optical faces is prevented by a rapid flow of precooled, predried N_2 or Ar aimed at the points of entry and exit of the light beam.

As mentioned above, the protonic activity, often referred to as pa_H, is set in the cryosolvent at room temperature. Douzou et al. (1976) have determined the protonic activity in several buffer systems as a function of temperature and initial (room temperature) pa_H values using a variety of cryosolvent systems. These data are used to determine the protonic activity at low temperature in our reaction mixtures.

Formation of Cu(II)-Free DAO. The Cu(II) can be removed from DAO by the addition of 20 mM diethyldithiocarbamate (DDC; Sigma Chemical Co.). As the Cu(II)–DDC complex is insoluble in water, it can be removed by centrifugation at 100000*g* (Beckman Model L-50). Exhaustive dialysis of the apoenzyme is necessary to remove the excess chelating agent. Spectra of the resolved Cu(II)–DDC complex are obtained by dissolving the precipitate in CCl_4. This complex is characterized by an absorption maximum at 435 nm.

Results

Native Enzyme Spectrum. The visible spectrum of native pea seedling DAO is characterized by a single, broad absorption centered at 525 nm, which makes the enzyme visibly pink. This band has an extinction coefficient at 525 nm of about 1200 M^{-1} cm^{-1}.

Reaction of DAO at Subzero Temperatures. The visible spectrum of 2 mg/mL DAO (0.012 mM) was monitored after the addition of substrate at subzero temperatures. The cryosolvent was 40% Me_2SO in water, buffered with 0.05 M P_i. After obtaining a spectrum of the native enzyme at low temperature, we added *n*-butylamine to a final concentration of 20 mM.

The results obtained at −8 °C are shown in Figure 1. Curve A is the native enzyme spectrum. Curve B is the spectrum obtained immediately after the addition of substrate. After ~1 h, spectrum C was obtained. The disappearance of the 475-nm band of curve B occurs simultaneously with the appearance of the new bands at 463, 455, 432, and 360 nm, with an isosbestic point at 470 nm. The insert in Figure 1 shows the effect of removing the large protein absorption that extends into the visible region. This insert also shows the resolved spectrum of the native enzyme (curve E).

Figure 2 shows the 400–500-nm portion of individual spectra leading from curve B to curve C as in Figure 1, but at −5 °C. The time elapsed between spectra is 2 min. The absorbance at 463, 455, and 432 nm is plotted vs. time in the frame to

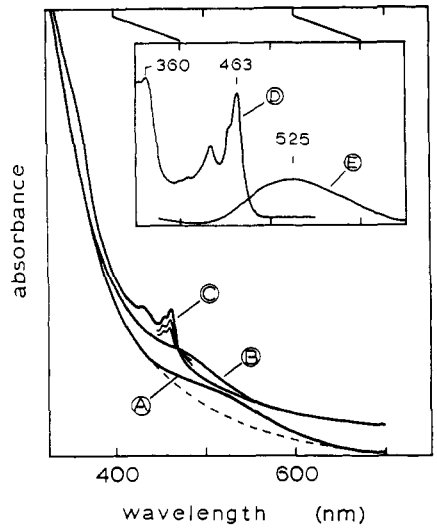

FIGURE 1: Spectra of DAO reacting at −8 °C. Curve A represents the visible spectrum of DAO in 40% Me_2SO and buffer at −8 °C; curve B was obtained immediately following the addition of 20 mM *n*-butylamine, while curve C was generated after about 1 h. Curves D and E in the insert show the effect of removing the large protein absorbance from curve C and from curve A (native enzyme), respectively.

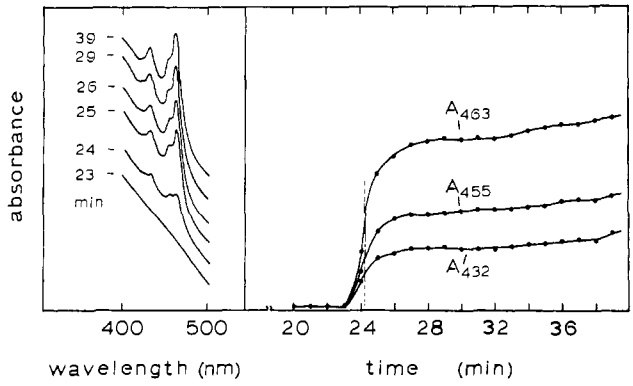

FIGURE 2: Formation of the E′ form of DAO at −5 °C. (Left frame) Shown are the individual spectra obtained from 23 to 39 min following the addition of *n*-butylamine at −5 °C. (Right frame) The increase in absorbance vs. time is plotted for the three absorption bands. The dashed vertical line indicates the time for 50% of the total absorption increase to occur; the half-life for formation of all three bands appears to be the same (about 1 min).

the right of the spectra. One can see that the half-life of the appearance of these three bands is the same (~1 min). Although its rate of appearance was not measured exactly, it appears that the 360-nm band also forms at the same time.

In the reaction of DAO with *n*-butylamine initiated at −15 °C, it appeared that the 525-nm band was lost, leaving a featureless spectrum that remained for at least 1 h. Upon rapid raising of the temperature to −7 °C, the 475-nm band appeared, was apparent for a short time, and again dissappeared with the formation of the previously mentioned four new bands. At −15 °C, the conversion of the 475-nm band to the set of four bands occurred with a half-life of ~3 min, beginning after 2 h.

Similar low-temperature reactions were run by using benzylamine and the synthetic substrate DAB; while the kinetic parameters were not obtained, we found that the same spectrum as that in Figure 1, curve C, was obtained, independent of which substrate was used.

Spectrum of Anaerobic DAO. Freeze-dried DAO was dissolved in degassed buffer and further swept with Ar in a

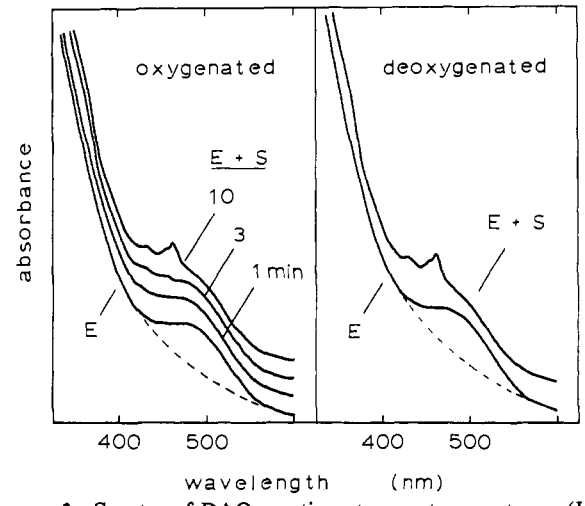

FIGURE 3: Spectra of DAO reacting at room temperature. (Left frame) The spectra shown were obtained at various times following the addition of *n*-butylamine to a solution of DAO saturated with O_2. (Right frame) These spectra were obtained under the same conditions with the exception that the solution is highly O_2-depleted.

sealed spectrophotometer cell. (The spectrum of the enzyme obtained was identical with that when O_2 was present.) Upon the addition of substrate, the enzyme immediately turned from pink to yellow. The visible spectrum of this species revealed the same set of bands as shown in Figure 1, curve C and the insert (curve D). Therefore, the enzyme form generated after reaction at low temperature appears to be the intermediate, reduced E′ form.

Dithionite Reduction of Cu(II) to Cu(I). Solutions of both native and reduced (E′ form) DAO were made 20 mM in $Na_2S_2O_4$. This reagent immediately bleached both the pink and yellow colors, respectively, of these solutions. Visible spectra revealed that the 525-nm band was gone from the native enzyme spectrum, as were the 463-, 455-, 432-, and 360-nm bands of the E′ form.

Spectra of DAO Reacting under Steady-State Conditions. In order to examine the relative rates of the complete anaerobic and aerobic stages of the DAO reaction, we allowed the reaction of DAO with *n*-butylamine to reach a steady state at room temperature, at which time visible spectra were obtained. Under conditions of O_2 depletion (not totally anaerobic but partially O_2 starved), the visible spectrum is a "hybrid" of the spectra of the native and E′ forms, with the larger component apparently being the contribution of the E′ form. This indicates a substantial accumulation of this form of the enzyme due to the aerobic stage being relatively slow. However, under conditions of O_2 saturation, the spectrum of the reacting enzyme is identical with that of the native enzyme (with the exception that the pink band is centered closer to 475 nm than 525 nm). After the reaction had proceeded for some time, so that the O_2 concentration was reduced, the bands characteristic of the E′ form of DAO again appeared. These spectra are presented in Figure 3.

It is interesting that upon addition of $Na_2S_2O_4$ to the above solutions, we find that the set of spectral bands characteristic of the E′ form of DAO again disappear immediately; however, in contrast to the native enzyme, the 475-nm portion of the hybrid spectrum is not bleached until 1–2 h following the addition of the reducing agent.

Cu(II)-Free Native and Reduced DAO. Still more spectral information is obtained when the metal ions are removed from the active site. When Cu(II) is removed from native DAO, two new absorption bands appear, at 395 and 410 nm. Presumably these represent the organic cofactor, while the original

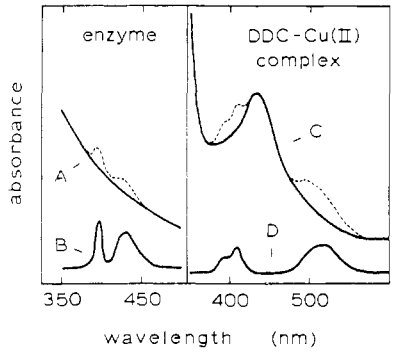

FIGURE 4: Spectra of Cu(II)-free DAO and DDC–Cu(II) complexes. (Left frame) Curve A, including the dashed-line portions, represents the visible spectrum of Cu(II)-free native DAO. The E′ form of DAO without Cu(II) shows only the solid-line portion of curve A. Curve B shows the two peaks of native Cu(II)-free DAO resolved from the protein-absorption base line. (Right frame) The spectrum of the DDC–Cu(II) complex isolated from native DAO is depicted by the solid-line portion only of curve C, while that isolated from DAO in its intermediate (E′) state also includes the three bands represented by the dashed lines. These three additional peaks are shown resolved from the main DDC–Cu(II) band (centered at 435 nm) in curve D. [Solvent for the DDC–Cu(II) complexes was CCl_4.]

525-nm band reflects some combination of the two cofactors. However, when DDC is added to the reacting enzyme, which is in its intermediate (E′) state, and the resulting metal–chelate complex is removed, the spectrum of the enzyme is featureless (Figure 4), indicating that the organic cofactor is removed along with Cu(II). This is further verified by the spectra of the resolved chelate complexes. The spectra of these two species (in CCl_4) obtained from the native and reacting enzymes are also shown in Figure 4. The spectrum of the DDC–Cu(II) complex from the native enzyme is indistinguishable from that of the authentic complex with no other ligands (other than water). However, the spectrum of the species obtained from the reacting system shows two new absorption bands near 400 nm [in fact, they are quite similar to the two bands observed in the native enzyme from which Cu(II) had been removed].

Discussion

It is apparent from the above results that one obtains substantially more spectral information, in particular about the organic cofactor of DAO, if one observes the enzyme in its intermediate (E′) state. Several conclusions can be drawn from the low-temperature spectra. The E′ form of DAO is apparently formed via at least one or two other intermediates, as evidenced by the initial loss of the 525-nm band, the appearance of a similar band centered closer to 475 nm, and, finally, the appearance of the new set of four bands. These new bands all appear at the same rate and after the same lag period. In addition, the spectrum obtained at this time is identical with that obtained by anaerobic conversion of DAO into the E′ state. The fact that this spectrum is independent of substrate suggests that it does in fact represent a free-enzyme form. Hill & Mann (1964) had initially interpreted their observation of yellow forms of DAO as resulting from complexes of substrate with enzyme, which is apparently not correct. Further examination of the reaction of DAO at subzero temperature is in order, as the lag period observed before the formation of the yellow form of the enzyme remains unexplained.

The values of the extinction coefficient for the absorption bands giving rise to the pink color of native DAO and the yellow color of the E′ form of DAO suggest that there are interactions which are charge transfer in nature in *both* forms of the enzyme. We propose that there is a substantial interaction of the Cu(II) with the organic cofactor even in the native enzyme.

While the intermediate state of DAO is in a sense a "reduced" form, having not bound O_2, one can observe the system containing Cu(I) by reduction with dithionite. Reduction of the metal ion results in a disappearance of both the pink color of the native enzyme and the yellow color of the intermediate DAO. This result lends further support to the observation that Cu(II) is not reduced to Cu(I) during the catalytic cycle. The loss of all visible absorption bands after dithionite reduction also indicates that these spectral bands reflect both the organic cofactor and the Cu(II), with which it interacts.

One can examine the relative amounts of DAO in the native and E′ forms as it reacts under steady-state conditions at room temperature. The spectra shown in Figure 3 indicate that both of these species are present; as one might suspect from the ping-pong reaction mechanism, the amount in the intermediate form depends on the O_2 concentration. This was found to be the case. However, we also observed that unless O_2 is present at nearly a saturation level, the aerobic stage of the reaction is at least partially rate limiting and the spectrum shows a buildup of the E′ form. The fact that the component of the hybrid spectra of Figure 3 near 475 nm is lost at a very low rate upon the addition of dithionite may be due to the fact that the presence of amine, perhaps in the form of the initial Michaelis complex, protects the Cu(II) from ready reduction. (Recall that this reagent bleached both the pink and yellow colors of DAO *immediately* in the nonreacting case.)

Finally, it appears that the interaction between the organic and metal ion prosthetic groups is much stronger in the E′ form of DAO; that is, a part of the organic group is actually bonded to Cu(II). Our recent NMR study of reacting DAO indicated that a water ligand is lost upon conversion of DAO to its intermediate state, lending further support to this conclusion. When Cu(II) is removed from the E′ form of DAO, not only is the resulting enzyme spectrum featureless (as opposed to that found when native DAO is freed of metal) but also the resolved chelate complex spectrum indicates the presence of a new ligand to Cu(II). In addition, the four resolved curves comprising the spectrum of this complex (Figure 4) appear to be very similar to the absorption band at 525 nm of native DAO, those at 395 and 410 nm of Cu(II)-free native DAO, and, of course, the 435-nm band of authentic DDC–Cu(II). It appears that we have an easy method of removing the organic moiety; such a method has not previously been found. Work on further characterization of this chelate complex and on the identification of the organic cofactor so isolated is currently underway in our laboratory.

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Interaction of Phospholipase A_2 from Cobra Venom with Cibacron Blue F3GA†

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ABSTRACT: Cibacron Blue F3GA has been suggested as a site-specific probe for dinucleotide binding sites and the "dinucleotide fold" in proteins [Stellwagen, E. (1977) *Acc. Chem. Res. 10*, 92]. However, we have found that cobra venom phospholipase A_2 (*Naja naja naja*) reversibly binds the Cibacron Blue dye with a $K_d \simeq 2$ μM as measured by difference spectroscopy. NADH and NAD^+ will not displace the dye from phospholipase A_2, but the water-soluble phospholipid dihexanoylphosphatidylcholine will. The dye inhibits catalysis, and a double-reciprocal plot of inhibition as a function of dye concentration is linear and yields a $K_i \simeq 3.5$ μM. *p*-Bromophenacyl bromide chemically modifies the active site of phospholipase A_2, and the Cibacron dye inhibits this process with an apparent $K_d \simeq 7$ μM. When the dye–enzyme interaction is monitored at low protein concentrations (less than 2 μM), the difference spectral titrations, inhibition of catalysis, and prevention of chemical modification by *p*-bromophenacyl bromide all suggest that the dye interacts with a single type of site on the phospholipase A_2. However, at higher protein concentrations where cobra venom phospholipase A_2 is known to exist as dimers and higher order oligomers, the difference spectra show the appearance of new types of binding sites. These data demonstrate that Cibacron Blue F3GA is not a reliable, specific probe for the dinucleotide fold in proteins and that the dye is a useful probe for exploring the dimerization of phospholipase A_2 and phospholipid binding to the enzyme.

Stellwagen (1977) has reported that many dehydrogenases and kinases bind tightly to immobilized Cibacron Blue F3GA dye. According to Stellwagen (1977), this interaction is sufficiently specific that it constitutes a diagnostic test for a supersecondary structural feature in enzymes known as the "dinucleotide fold". The reaction catalyzed by phospholipase A_2 (EC 1.1.1.4) (Deems & Dennis, 1975) does not involve dinucleotides, mononucleotides, or sugar phosphates, and it is highly unlikely that this enzyme contains a dinucleotide fold. Yet, we have found that cobra venom phospholipase A_2 binds quite tightly to Blue Dextran, a conjugate of Cibacron Blue F3GA and dextran used as a void volume marker in gel filtration experiments. We report here an investigation of the binding of the free dye Cibacron Blue F3GA to cobra venom phospholipase A_2 by utilizing primarily spectroscopic methods. These studies suggest that the dye binding site on phospholipase A_2 includes the active site of the enzyme and that this provides a somewhat different environment for the dye than does the dinucleotide fold of dehydrogenases and kinases.

Experimental Procedure

Lyophilized cobra venom, *Naja naja naja* (Pakistan), Lot NNP8STLZ, was obtained from the Miami Serpentarium. Phospholipase A_2 was purified according to the procedure of Deems & Dennis (1975, 1980). The protein concentration was determined by the method of Lowry et al. (1951). Enzymatic activity was measured by the pH stat procedure carried out at 40 °C using mixed micelles of Triton X-100 and egg phosphatidylcholine as substrate (Dennis, 1973). The egg phosphatidylcholine was purified by the method of Singleton et al. (1965). Dihexanoylphosphatidylcholine[1] and octanoyl- and myristoyllysophosphatidylcholine were purchased from Calbiochem. All other chemicals were reagent grade.

Cibacron Blue F3GA was a generous gift from Dr. H. Bosshard of Ciba-Geigy, Basel, Switzerland. Weber et al. (1979) have recently reported that commercial samples of Cibacron Blue F3GA contain contaminants, one or more of which may irreversibly inhibit an enzyme. The difference spectroscopy experiments described here were conducted with dye that was purified by chromatography on silica gel (60–200 mesh) as described by Weber et al. (1979). The binding of Cibacron Blue F3GA to the enzyme was detected by measuring difference spectra in the visible region (450–800 nm) following the procedures of Thompson & Stellwagen (1976). Difference spectra were recorded at room temperature with a Cary 219 double-beam spectrophotometer using 1-cm path length cells. When low protein concentrations were used, a Cary 118 double-beam spectrophotometer and 10-cm path length cells were employed. The dye concentration was determined spectrophotometrically at 610 nm by using a molar

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[1] Abbreviations used: diacylphosphatidylcholine, 1,2-diacyl-*sn*-glycerol-3-phosphorylcholine; acyllysophosphatidylcholine, 1-acyl-*sn*-glycerol-3-phosphorylcholine.

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