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Fluorescence Properties of Reconstituted Forms of UDP-Glucose 4-Epimerase from *Saccharomyces fragilis* *

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UDP-glucose 4-epimerase from *Saccharomyces fragilis* exhibits a very characteristic intense fluorescence with an excitation maximum at 360 nm and an emission maximum at 433 nm. The fluorescence spectrum resembles the fluorescence of free NADH with an apparent blue shift and, although the exact nature of the fluorophore is not known, the protein-bound NAD, which is a coenzyme for this reaction, or its reduced form is obviously involved in the emission of the fluorescence. The fluorophore therefore constitutes part of the active site.

The inactivation of epimerase with diazinedicarboxylic acid bis(*N,N*-diethylamide), a reaction shown in the previous paper to form a disulfide linkage across the subunits, results in a simultaneous and correlated loss of the characteristic fluorescence of the enzyme. Reaction with mercaptoethanol restores the native fluorescence with a 2 nm blue shift in emission maximum. These experiments provide additional evidence that the two conformationally vicinal sulfhydryl groups are located at the active site. Unlike the reconstituted enzyme obtained from the diamide-inactivated enzyme, the partially active enzymes reconstituted from *p*-chloromercuribenzoate-inactivated and heat-inactivated enzymes fail to show the reappearance of the characteristic native fluorescence. Treatment with *N*-ethylmaleamide, on the other hand, leads to a form of the inactive enzyme that fully retains its fluorescent properties. A model depicting the minimal changes at the active site during the process of inactivation and reconstitution by these various treatments is presented.

UDP-glucose 4-epimerase catalyzes a freely reversible reaction between UDP-glucose and UDP-galactose in a wide variety of cells (1). The enzyme has been purified extensively from *Escherichia coli* (2), *Saccharomyces fragilis* (3), calf liver (4, 5), and bovine mammary gland (5). In all cases, NAD is an obligatory participant in the catalytic process, although its mode of association with the apoenzyme varies widely in enzymes isolated from different sources. Thus, while the liver and the mammary gland enzymes need exogenous NAD for catalytic activity, the *E. coli* and the yeast enzymes have a mole of NAD bound per mol of the active apoenzyme. However, a very interesting and important difference exists between the yeast and the *E. coli* enzymes. The yeast enzyme has a unique fluorescence with an excitation maximum at 360 nm and emission maximum at 433 nm which closely resembles the fluorescence of native NADH, excepting that a significant blue shift is observed in this case (6). Free NADH in an aqueous solution, pH 8.3, has excitation maximum at 340 nm and emission maximum at 457 nm (7).

The highly characteristic fluorescence is related clearly to the bound nucleotide, since reduction of the nucleotide on the enzyme surface by chemical (8, 9) or by enzymatic methods (10, 11) resulted in greatly enhanced fluorescence of the holoenzyme. Apparently, either NAD in combination with some amino acid residues or a population of enzymes containing NADH, as assumed by Kalckar and his group (1), is responsible for this intense fluorescence. This characteristic native fluorescence of the yeast enzyme will be designated, henceforth as the coenzyme-fluorescence in this text. Since pyridine nucleotide is involved both in the catalysis and in the fluorescence, it is obvious that whatever be the nature of the fluorophore, it must constitute part of the active site of the yeast enzyme. Modification of the amino acid residues of the enzyme and simultaneous monitoring of this characteristic native fluorescence may therefore provide a useful means of exploring the conformation and the role of specific amino acid residues at the active site of the enzyme. The only essential precondition for such fluorescence studies with modifying reagents is that the pyridine nucleotide must be retained on the enzyme surface, since release of the cofactor from the apoenzyme renders the protein completely nonfluorescing or "dark," as observed earlier by many workers (6, 8, 12).

In the preceeding paper, we showed that treatment of the yeast enzyme with diamide, a diazene derivative that specifically oxidizes conformationally vicinal sulfhydryl groups to disulfide bonds, resulted in a form of the enzyme that lost its activity but still retained the quarternary structure and also the pyridine nucleotide at the active site (13). The diamide-treated inactive enzyme could be reactivated in presence of mercaptoethanol. The yeast enzyme could also be reactivated partially from other inactive forms of the enzyme that were obtained either by treatment with *p*-chloromercuribenzoate (6, 8) or by exposure to controlled heat (14). All these inactivated enzyme species are nonfluorescent. When fluorescence properties of the various reconstituted forms of the enzyme were monitored, only the diamide-inactivated enzyme was found to have regained the characteristic native fluorescence. Prior to this report, all attempts to regenerate fluorescence in the nonfluorescent inactive epimerase were always unsuccessful. Obviously, subtle but significant conformational differences exist at the active site of these reconstituted enzymes. Moreover, a possible involvement of one or more sulfhydryl groups in constituting the fluorophore for the characteristic native fluorescence of the yeast enzyme was revealed during the course of this work. Finally, these fluorescence studies have provided strong supportive evidence to indicate the presence of the two sulfhydryl groups at the active site region of the enzyme.

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MATERIALS AND METHODS

All biochemicals were purchased from Sigma Chemical Co. St. Louis, MO. Diamide was taken freshly into solution in water for each set of experiments. UDP-glucose 4-epimerase was either purified in the laboratory or purchased from Sigma Chemical Co. The enzyme was assayed according to the methods described in the previous paper (13).

Fluorescence was measured with a Perkin-Elmer Spectrofluorimeter model MPF 44A. In all cases of fluorescence measurements, the samples were excited uniformly at 360 nm. When the enzyme was dissolved in 0.05 M glycylglycine buffer, pH 7.8, the solvent itself was found to have a very minor residual fluorescence. No attempt was made to remove this contaminating fluorescence by recrystallization of glycylglycine and removal of contaminating organic substances. Diamide in the same buffer did not have any native fluorescence of its own.

RESULTS

Effect of Diamide on the Coenzyme Fluorescence—In the previous paper, we had shown that on treatment with diamide the yeast epimerase gradually lost its activity and the activity could be almost fully restored on treatment with mercaptoethanol. When the fluorescence of the enzyme was monitored under these conditions, the fluorescence was found to decrease gradually and was virtually abolished in 120 min (Fig. 1). Immediately after addition of 2 mM diamide, there was an instantaneous and substantial decrease in fluorescence (about 50%) (Fig. 1*B*) which was possibly due to an inner filter effect (15). The spectrum of diamide showed that substantial light was absorbed at 360 nm, which is the excitation wavelength for monitoring the coenzyme fluorescence. When 8 mM mercaptoethanol was added to the nonfluorescing enzyme species, the enzyme rapidly regained about 70% of its native fluorescence. The emission maximum for this regained fluorescence was found to be 431 nm. The reason or implication for this slight blue shift in emission maximum (from 433 nm to 431 nm) was not explored. Control experiment with diamide and mercaptoethanol in absence of the enzyme did not show any fluorescence in this region. Diamide had been reported previously to oxidize NADH rapidly (16). When 5 μM NADH in glycylglycine buffer, approximately equivalent to the molar amount of total pyridine nucleotide present in the apoenzyme, was allowed to react with 2 mM diamide, the 450 nm fluorescence disappeared within 2 min, indicating oxidation of free NADH to NAD. When mercaptoethanol (8 mM) was added to this solution, no regeneration of fluorescence was observed. A further control, consisting of NAD, diamide, and mercaptoethanol also failed to show emergence of any fluorescence at this region. Obviously, abolition of coenzyme fluorescence and its regeneration is dependent on formation of disulfide bond(s) with diamide and its subsequent reduction with mercaptoethanol. Exclusive dependence on mercaptoethanol for the re-emergence of fluorescence and also for restoration of activity for the diamide-inactivated enzyme is more clearly shown, when excess diamide was removed from the nonfluorescent inactive enzyme by passing the inactivated enzyme through a Sephadex G-50 column (Fig. 2).

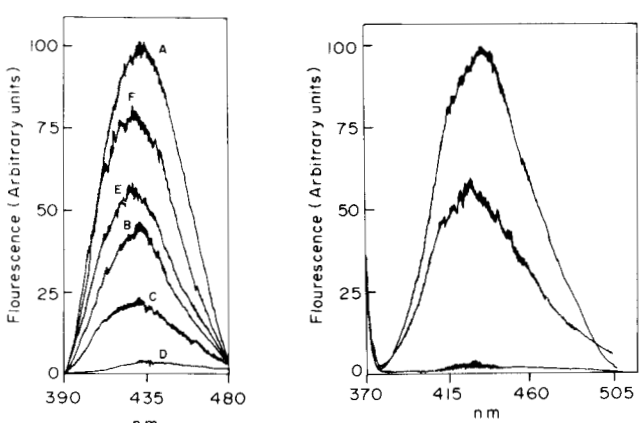

FIG. 1 (*left*). **Effect of diamide on coenzyme fluorescence.** For this experiment, 0.8 mg of the epimerase was taken in 1 ml of 0.1 M glycylglycine buffer, pH 7.8. Other experimental conditions are described in the text. *A*, native enzyme; *B*, *C*, and *D* are 2 min, 30 min, and 120 min after addition of diamide, respectively. *E* and *F* are tracings 3 min and 10 min after addition of mercaptoethanol to the diamide-treated enzyme (120 min).

FIG. 2 (*right*). **Regeneration of fluorescence of the diamide-treated enzyme after removal of excess diamide.** Details of the experiments are described in the text. *Top tracing,* native epimerase; *lower tracing,* diamide-treated enzyme; *middle tracing,* diamide-treated enzyme, 5 min after addition of mercaptoethanol.

For this experiment, 4 mM diamide was added to 0.4 mg of protein in 0.2 ml of 0.05 M glycylglycine buffer, pH 7.8. After an incubation period of 2 h, both the activity and the fluorescence of the enzyme were checked. The diamide-treated enzyme was found to have less than 2% of its original activity and the coenzyme fluorescence of the enzyme was abolished completely. The residual trace fluorescence was due to contaminating glycylglycine buffer as was evidenced by a control buffer solution which did not contain the protein. The inactivated nonfluorescing enzyme was now passed through a Sephadex G-50 column (0.8 × 20 cm). When 5 mM mercaptoethanol was added to the diluted, diamide-free enzyme the coenzyme fluorescence was regenerated rapidly. The maximum fluorescence was obtained within 5 min of addition and prolonged incubation did not result in any further increase in fluorescence. For comparison, an equal amount of untreated enzyme was also passed through a similar column and the extent of regeneration was compared against the native fluorescence. About 60% of the fluorescence, was regenerated on treatment with mercaptoethanol (Fig. 2). When specific activity and specific fluorescence (fluorescence at emission maximum per mg of protein) of the native enzyme were compared with the specific activity and specific fluorescence of the regenerated enzyme, 71% of activity and 63% of the fluorescence was found to be restored.

Correlation between Activity and Fluorescence—Since on treatment with diamide the enzyme lost both its activity and its fluorescence, it was important to find out if there was any direct correlation between loss of activity and loss of fluorescence. When such an experiment was conducted a striking correlation between the two was observed (Fig. 3). For this experiment, 0.42 mg of the enzyme was taken in 0.4 ml of 0.05 M glycylglycine buffer, pH 7.8, in a fluorimeter cell. Diamide was added to a final concentration of 2.5 mM. During the course of the experiment, the fluorescence of the sample was recorded directly. For assay of the enzyme, 5-μl aliquot of the enzyme was transferred to 400 μl of 0.1 M glycylglycine buffer, pH 7.8. This allowed diamide to be diluted substantially so that it did not have any further effect on the activity of the enzyme and the activity could be assayed at any subsequent time. Suitable aliquot from this diluted medium was then taken for the final assay of the enzyme. Immediately after addition of diamide there was a substantial decrease (about 65%) in fluorescence due to "inner filter" effect. The enzyme activity however was affected by less than 4%. The fluorescence and the enzyme activity at this point of time were taken to be 100% each and later values were expressed with regard to these values. Fig. 3 indicates that a very good correlation exists between the rate of inactivation and loss in fluorescence. A good correlation was also observed between the rate of regeneration of activity and rate of restoration of fluorescence (data not shown). Obviously, oxidation of the sulfhydryl

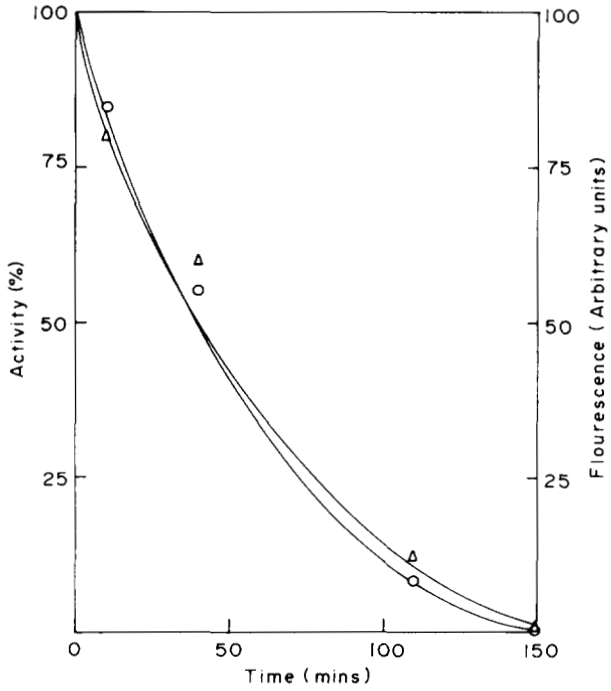

FIG. 3. **Correlation between activity and fluorescence on treatment with diamide.** O——O and Δ——Δ are activity and fluorescence of the enzyme, respectively. Fluorescence emissions at 433 nm are measured. Other details of the experiment are described in the text.

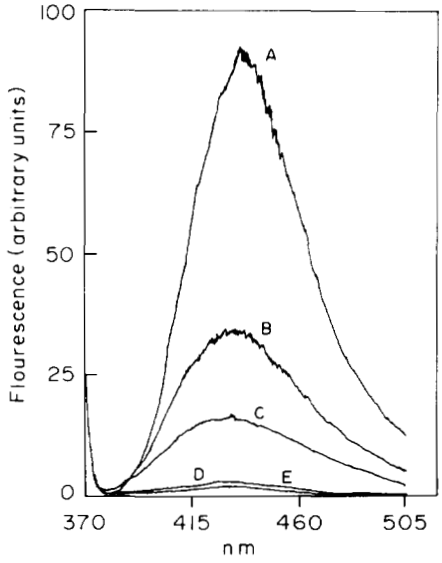

FIG. 4. **Fluorescence of epimerase on exposure to heat.** For this experiment, 1 mg of the epimerase was taken in 1 ml of 0.1 M glycylglycine buffer, pH 7.8. The enzyme solution was heated at 45°C. Fluorescence and activity of the enzyme were monitored from time to time. *A*, native enzyme; *B*, *C*, and *D* are after 10 min, 15 min, and 20 min of heat treatment, respectively. *E* is the fluorescence tracing 30 min after addition of mecaptoethanol (5 mM) and NAD (0.5 mM) to 20-min heat-treated enzyme.

groups leading to formation of disulfide bridge affected the catalytic activity and the fluorescence properties of the fluorophore to equal extent. Previously, by stepwise titration of the total sulfhydryl groups of the enzyme with *p*-chloromercuribenzoate, Creveling *et al.* (8) could show that the loss of activity and the loss in fluorescence followed two completely different paths. Evidently, not all sulfhydryl modifications of the enzyme affect the fluorescence and the catalytic properties in the same way.

Fluorescence Properties of the Heat-inactivated Enzymes—We had earlier reported that controlled heating at 45°C, resulted in a form of inactive enzyme, which could be partially reactivated when incubated in presence of mercaptoethanol and exogenous NAD (14). When the coenzyme fluorescence was monitored for this process, the enzyme was found to gradually lose its fluorescence which, however, could not be restored on reactivation of the enzyme (Fig. 4). In this case, although about one-third (30%) of the activity was restored, no corresponding regeneration of fluorescence was observed. As in the case of the reconstituted enzyme after its inactivation with *p*-chloromercuribenzoate, here also a form of the reconstituted enzyme is obtained which shows partial activity but no characteristic native fluorescence. When loss of activity and loss of fluorescence were monitored simultaneously at different intervals of time for the heat inactivation process, a reasonably good correlation between the two was observed (Table I). Compared to our earlier result (14), a longer time (about 20 min) was needed for complete inactivation of the enzyme. This was probably due to the fact that for this experiment higher concentration of the protein was used.

TABLE I

Correlation between activity and fluorescence of the enzyme during heat treatment

For this experiment, 2.0 mg of enzyme was taken in 2 ml of 0.1 M glycylglycine buffer, pH 7.8. The enzyme solution was heated to 45°C in a water bath. Aliquots (0.4 ml) were removed at specified time intervals and then cooled rapidly on an ice bath. Fluorescence of the samples were measured directly at 433 nm (emission maximum) and activity of the samples were measured by taking suitable aliquots.

	Activity	Fluorescence
	%	%
Native enzyme	100	100
5-min heated enzyme	42	51
10-min heated enzyme	22	30
15-min heated enzyme	14	22
20-min heated enzyme	2	4

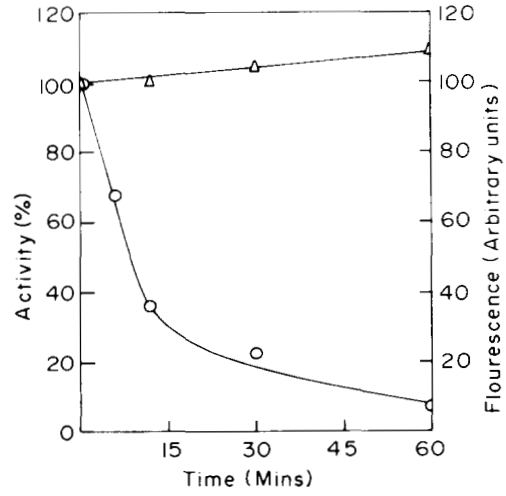

FIG. 5. **Fluorescence and inactivation of epimerase with *N*-ethylmaleimide.** For this experiment, 0.32 mg of the epimerase was taken in 0.4 ml of 0.05 M glycylglycine buffer, pH 7.8, in a fluorimeter cuvette. *N*-Ethylmaleimide was added to a final concentration of 1 mM. During the course of the experiment, the fluorescence of the sample at 433 nm (emission maximum) was recorded directly at different time intervals. For assay of the enzyme, 5-μl aliquot of the enzyme was transferred to 400 μl of 0.1 M glycylglycine buffer, pH 7.8, at indicated time intervals. Suitable aliquots from these diluted samples were taken for final assays of the enzyme. O——O and Δ——Δ stand for activity and fluorescence of the epimerase, respectively, after *N*-ethylmaleimide treatment.

Modification of the enzyme with controlled heat treatment resulted in the cross-linking of the subunits through freshly formed disulfide bond(s). This was evidenced by the fact that the heat-inactivated enzyme dimer could not be dissociated into monomers when sodium dodecyl sulfate-gel electrophoresis was carried out in absence of mercaptoethanol. The reactivated enzyme, on the other hand, dissociated into monomers in the same way as the native enzyme (data not shown). The sodium dodecyl sulfate-gel electrophoretic pattern obtained with the heat-inactivated and reactivated enzymes were of the same pattern as obtained for the diamide-treated enzyme.

Inactivation with N-Ethylmaleimide—Our earlier works with 5,5′-dithiobis(2-nitrobenzoate) (17) and also with diamide (13) had shown the presence of highly reactive sulfhydryl groups at the active site of the yeast epimerase. When the enzyme was treated with *N*-ethylmaleimide (1 mM), the enzyme was found to lose its activity rapidly, but interestingly enough, the fluorescence of the enzyme remained completely unaffected (Fig. 5). In fact, in this case, a very slow but definite increase in fluorescence was observed. *N*-Ethylmaleamide-treated enzyme was thus a form of the enzyme that retained full fluorescence but lost all catalytic activity. The situation is in sharp contrast to the other forms of the inactivated enzymes which are invariably "dark" or nonfluorescing in nature. Further analysis of number of sulfhydryl groups titrated or their relation to the active site of the enzyme on treatment with *N*-ethylmeleimide was not carried out. Attempts to reactivate the enzyme with mercaptoethanol and NAD were not successful.

DISCUSSION

The monitoring of the coenzyme fluorescence for the inactivated and reconstituted enzymes reveals certain interesting features of the enzyme. In the previous paper, we had shown that on oxidation with diamide, only one disulfide linkage is formed across the subunits and this results in complete inactivation of the enzyme. Apparently, two sulfhydryl groups, each residing in the two separate subunits, constitute part of the active site. This was evidenced by the partial protection provided by the substrates against inactivation with diamide. A more conclusive demonstration that the sulfhydryl groups are part of the active site is provided by the fluorescence experiments (Figs. 2 and 3). The diamide-treated inactive enzyme still retains the coenzyme in the active site and the slow abolition of the coenzyme fluorescence is obviously due to the formation of the disulfide bond. The rapid regeneration of fluorescence on reduction of the disulfide bond with only mercaptoethanol conclusively establishes the role of the disulfide bond in abolishing the fluorescence. Whatever be the mechanism for this loss of fluorescence, the two sulfhydryl groups involved in the formation of the disulfide bonds, must be in close physical proximity to the pyridine nucleotide fluorophore and hence must be in the domain of the active site of the enzyme.

As regards the nature of the fluorophore, there is no *a priori* reason to assume that some populations of the enzyme that presumably have NADH bound at the catalytic site are responsible for the fluorescence of the enzyme (1). No systematic analysis for the presence of NADH in the purified enzyme has been published. In fact, enzyme preparations have been obtained which are moderately fluorescent but do not show presence of any NADH by direct analysis (8). Further, apoenzymes containing reduced pyridine nucleotides should be catalytically inactive, since these will be unable to carry out the initial oxidation of the substrate. Thus, an inverse correlation would be expected between the activity and the fluorescence of the native enzyme. Purified, homogeneous enzyme preparations from different batches show substantial variations in their fluorescence which in no way correlates with the activities of the preparations (3, 8). Finally, inactivation with *p*-chloromercuribenzoate leads to complete abolition of the native fluorescence (6, 8). Since in this case the coenzyme is released in the medium, presence of free NADH in the medium should have been detected by fluorimetric methods and the titrated enzyme preparations should have shown some residual fluorescence. This, however, is not the case. The alternative possibility that NAD in combination with some amino acid residues at the active site constitute the fluorophore therefore should be considered seriously. NAD itself is nonfluorescing, but in the presence of strong alkali (18) or cyanide (19), it is known to fluoresce strongly. It is conceivable that one of the sulfhydryl groups in the form of a thiolate anion weakly interacts with C_4 of the pyridine ring to give a NADH-like structure which constitutes the fluorophore. Further enhancement of the fluorescence may then take place due to the hydrophobic milieu of the fluorophore and presence of neighboring tryptophan residues (6). Formation of disulfide linkage on oxidation with diamide will disrupt this structure and hence the resultant loss of the coenzyme fluorescence. In absence of any further evidence, however, this should be considered as basically speculative at the moment.

The minimum changes that take place at the active site and in the quarternary structure of the holoenzyme during various treatments can be depicted by the following model (Fig. 6). Treatments with *p*-chloromercuribenzoate, heat, and diamide result in three different forms of the inactive enzyme. Only in case of inactivation by *p*-chloromercuribenzoate, the dimer is dissociated and the coenzyme is released in the medium (20, 21). Both for heat treatment and for diamide inactivation (13) the dimeric structure is retained, but only in case of heat treatment the coenzyme dissociates from the apoenzyme (14). In both these cases, a disulfide bridge across the subunits is formed. These were evidenced by sodium dodecyl sulfate-gel electrophoresis in absence of mercaptoethanol for these two inactivated forms of the enzyme. The heat-inactivated enzyme, however, differs from the diamide-inactivated enzyme in that in this case the pyridine nucleotide dissociates from the apoenzymes and exogenous NAD is needed for reconstitution of the active enzyme (14).

All the reconstituted forms of the enzyme need mercaptoethanol for regeneration of critical sulfhydryl groups. The reconstituted form obtained from the diamide-inactivated enzyme (Form C) is obviously different from the reconstituted forms obtained from *p*-chloromercuribenzoate-inactivated and heat-inactivated enzymes (Forms A and B). This form shows almost full catalytic activity and very substantial regeneration of the native fluorescence. Therefore, Form C is closest to the native enzyme. Both Form A and Form B are about 35 to 40% active in comparison with the native enzyme and are completely nonfluorescing. By these two criteria, these two reconstituted forms seem to be identical. Apparently, once the cofactor is dissociated from the apoenzyme, restoration of the original conformation of the fluorophore can not be achieved very easily. Whether this is due to some irreversible denaturation of protein structure either by complete titration with *p*-chloromercuribenzoate or by heat treatment is not clear at the moment.

One fact, however, emerges clearly. Since Forms A and B are at least partially active and completely nonfluorescing, the conformational requirements at the active site, leading to the presence of the fluorophore, are not essential prerequisites for catalytic activity. Neither is catalytic activity an essential requirement for the existence of the fluorophore. This is

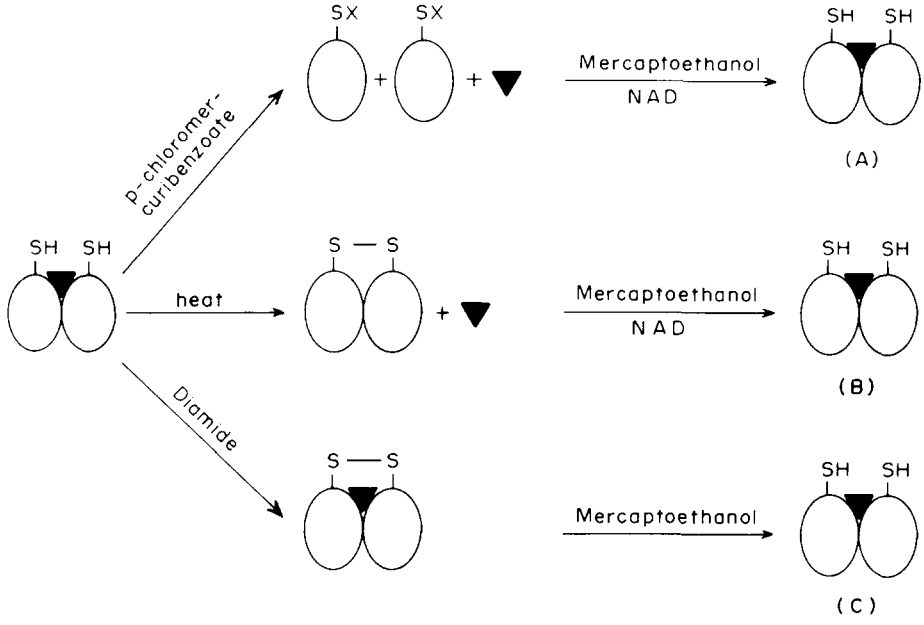

FIG. 6. **Model depicting minimal changes at the active site during various treatments.**

evident from the experiment with *N*-ethylmaleamide (Fig. 5) that generates a completely inactive but highly fluorescent form of the enzyme. It is possible that only one of the two sulfhydryl groups is essential for catalytic activity and the other one is essential for fluorophore structure. Only when both are modified simultaneously as in the case of diamide treatment, both the activity and the fluorescence are seen to be destroyed in a coordinated fashion (Fig. 3). Obviously, much more work needs to be done before the specific role of the two sulfhydryl groups at the active site either for generation of coenzyme fluorescence or for catalytic activity can be understood clearly.

If epimerase can be considered to be a subclass of racemases (22), it is interesting to note that two racemases, proline racemase and hydroxyproline racemase have been shown to have two sulfhydryl groups at the active site and both the sulfhydryl groups in both the enzymes seem to be closely involved in the catalytic process (23, 24).

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