Role of the Essential Thiol Groups of Yeast Alcohol Dehydrogenase

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1. Yeast alcohol dehydrogenase inactivated by reaction with iodoacetamide retains 85% of the original NADH-binding capacity as measured under conditions of saturating coenzyme concentration. 2. The dissociation constant of the enzyme–NADH complex is unaffected by inactivation of the enzyme with iodoacetamide, and the affinity of the enzyme for NAD^+ and pyridine-3-aldehyde–adenine dinucleotide ($PAAD^+$) appears to be similarly unaffected. 3. Enzyme inactivated with iodoacetamide has lost the ability to form normal ternary complexes of the type enzyme–NADH–acetamide and enzyme–$PAAD^+$–hydroxylamine that are characteristic of the native enzyme.

It has been observed for pig heart lactate dehydrogenase (Holbrook, 1966; Holbrook & Stinson, 1970) and glyceraldehyde 3-phosphate dehydrogenase (Friedrich, 1965) and for horse liver alcohol dehydrogenase (Li & Vallee, 1965) that modification of the essential thiol residues does not result in the loss of coenzyme binding capacity. In all cases, however, coenzyme binding is weakened. Studies with certain ternary complexes of lactate dehydrogenase indicate that inhibition of enzymic activity by modification of the essential thiol groups is due to the inability of the modified enzyme to form catalytic ternary complexes. With liver alcohol dehydrogenase, on the other hand, it appears that an abortive enzyme–NADH–ethanol complex can still be formed by the inactive enzyme.

Auricchio & Bruni (1969) have reported that yeast alcohol dehydrogenase still retains much NADH-binding capacity after inactivation by reaction with iodoacetamide or maleimide. Inactivation results from the blocking of four essential thiol groups/molecule (Whitehead & Rabin, 1964; Harris, 1964). It seemed worth while to investigate this system in more detail to find out, if possible, whether the strength of coenzyme binding is affected by blockage of the thiol groups and further, whether the inactivation of the enzyme is due to failure of treated enzyme to form catalytic ternary complexes or to a failure to catalyse hydride transfer within these complexes.

Materials and Methods

Materials

Water. All solutions were prepared with glass-distilled water.

Coenzymes. NAD^+ and NADH (fluorimetric grade) were purchased from Boehringer Corp.

(London) Ltd., London W.5, U.K. $PAAD^{+*}$ was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Iodoacetamide, acetamide and hydroxylamine. These were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. Iodoacetamide was recrystallized from 50% (v/v) ethanol and acetamide was recrystallized from methanol–ether. Hydroxylamine was obtained as the hydrochloride and solutions were neutralized to pH 7.0 before use.

1-Anilinonaphthalene-8-sulphonate. This was obtained as the sodium salt from Eastman Kodak Ltd., London, U.K., and after conversion into the magnesium salt was purified as described by Thompson & Yielding (1968).

Buffers. Unless otherwise stated all experiments were carried out in 0.1 M-sodium phosphate buffer, pH 7.0 and I 0.1, containing 0.3 mM-EDTA.

Enzyme. Alcohol dehydrogenase was prepared from baker's yeast (British Fermentation Products, Hull, U.K.) with slight modification of the procedure previously described (preparation I; Dickinson, 1970) or was purchased from the Boehringer Corp. (London) Ltd. The yeast currently obtained yields, when dried, the same quantity of enzyme activity but much less protein (25–30%) in the initial extract than did previous batches, which were obtained via local suppliers from a different source (Distillers Co. Ltd.). Because of this it was possible to decrease the size of the DEAE-cellulose column required in the purification to 2.5 cm × 50 cm. On application of the preparation to the column and after washing in 5 mM-phosphate buffer, pH 7.0, the enzyme was eluted directly in 40 mM-phosphate buffer, pH 7.0, at a concentration of about 5 mg/ml. After precipitation with $(NH_4)_2SO_4$ (390 g/l of eluate) the procedure reverted

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* Abbreviation: $PAAD^+$, pyridine-3-aldehyde–adenine dinucleotide.

to that already described. The yield of this procedure is generally slightly better than the earlier method but the specific activity of the product is the same.

Methods

Enzyme activity and protein concentration. Both of these quantities were determined as described previously (Dickinson, 1970).

Fluorescence titrations. These were performed at 25°C with a Farrand Mk. I spectrofluorimeter fitted with 20nm slits. For titrations involving 1-anilino-naphthalene-8-sulphonate together with NADH or other coenzyme, excitation was at 410nm with the analysing monochromator set at 550nm and a Corning 3-72 filter was included between sample and analysing monochromator. The settings served to minimize any contribution to the fluorescence measurements from NADH. In any case a slight correction was made for effects due to the intrinsic fluorescence of NADH. For titrations in the absence of 1-anilinonaphthalene-8-sulphonate, excitation was at 340nm with the analysing monochromator set to 470nm. A 1M-sodium nitrite filter was included between sample and analysing monochromator.

Gel-filtration studies. The binding of NADH by alcohol dehydrogenase was studied by the gel-filtration technique of Hummel & Dreyer (1962) as previously described (Dickinson, 1970). In this series of experiments about 150nmol of enzyme was used in each experiment and the concentration of NADH was varied within the range 7–340 μM. The column was maintained at 25°C. Concentrations and quantities of enzyme were calculated by using a molecular weight of 149000 (Dickinson, 1970).

Enzyme inactivation with iodoacetamide. Samples of enzyme (67–134 μM) were incubated at 25°C in phosphate buffer for 4h with excess of iodoacetamide with the ratio of initial concentrations [iodoacetamide]/[enzyme] in the range 0–8. At the higher iodoacetamide concentrations some precipitation of protein was noted by the end of the incubation period. With an eightfold excess of iodoacetamide about 10% of the protein was lost. In the experiments described below the inactivated enzyme was obtained by treatment with an eightfold excess of iodoacetamide unless otherwise stated. After inactivation the preparations were dialysed overnight and any small protein precipitate was then removed by centrifugation before determination of protein concentrations and enzyme activity.

Results and Discussion

Fig. 1 shows the fall in specific activity of solutions of yeast alcohol dehydrogenase (67 μM) after incubation for 4h at 25°C with various concentrations of excess of iodoacetamide. After 4h the reaction was
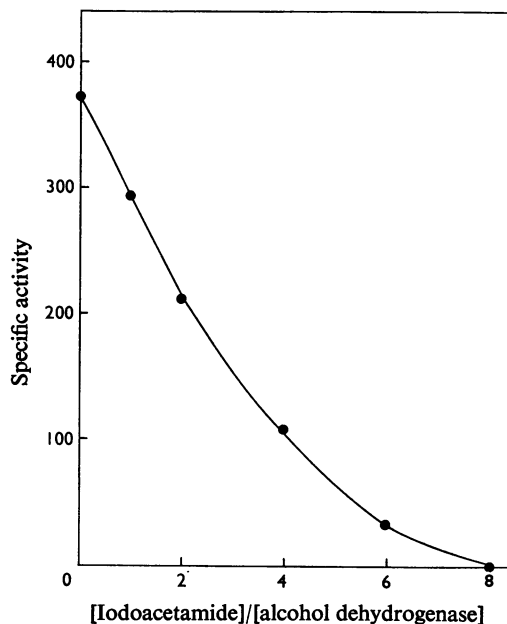

Fig. 1. *Inactivation of yeast alcohol dehydrogenase (67 μM) after incubation for 4h at 25°C with stated concentrations of excess of iodoacetamide*

Experimental details are given in the text.

complete and the remaining activity was stable under the prevailing conditions for a further 20h. Enzyme incubated under similar conditions but in the absence of iodoacetamide was stable for the whole incubation period.

It is apparent from Fig. 1 that reaction with an eightfold excess of iodoacetamide resulted in a loss of 99% of the total enzymic activity. The losses in activity were in direct proportion to the [iodoacetamide]/[alcohol dehydrogenase] ratio until a value of about 2.5 was attained. Above this ratio progressively increasing concentrations of iodoacetamide were required to produce equivalent losses in activity. Extrapolation from the initial slope of Fig. 1 yields an estimate of 4.5 thiol groups/molecule essential for the maintenance of enzymic activity. This result is in good agreement with those of other workers who have found that incubation of alcohol dehydrogenase with iodoacetamide or iodoacetate results in the preferential alkylation of 4 thiol groups/molecule which are essential for enzymic activity (Whitehead & Rabin, 1964; Rabin *et al.*, 1964; Harris, 1964). The present result is of some importance since recent work with alcohol dehydrogenase preparations has indicated a maximum of 3 coenzyme-binding sites/molecule (Dickinson, 1970), which is in contrast with the
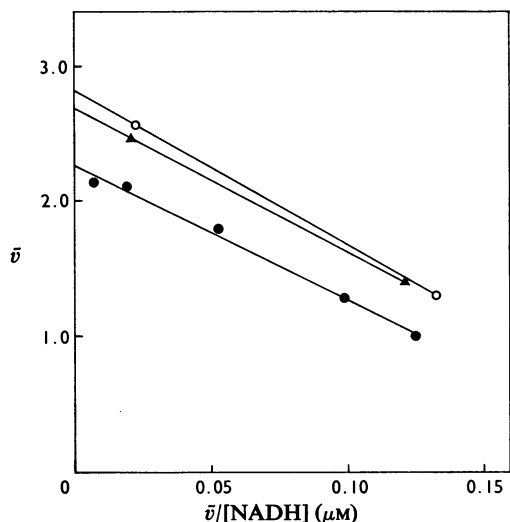
Fig. 2. *Binding of NADH by yeast alcohol dehydrogenase preparations at pH7.0 and 25°C*

Experimental details are given in the text. ▲, Freshly prepared enzyme; ●, freshly prepared enzyme inactivated with iodoacetamide; ○, commercial enzyme.

findings of other workers (Pfleiderer & Auricchio, 1964; Auricchio & Bruni, 1969). It seems that thiol-group reactivity is not concerned with this discrepancy.

Fig. 2 presents the results of titrations of native and inactivated alcohol dehydrogenase with NADH. The results were obtained by using gel-filtration chromatography according to the method of Hummel & Dreyer (1962) and are presented as a plot of the average number of molecules of NADH bound/molecule of enzyme ($\bar{v}$) versus this quantity divided by the concentration of free NADH ($\bar{v}$/[NADH]) in the mixture. It is evident that much NADH-binding capacity was retained by the inactivated enzyme. This observation confirms the findings of Auricchio & Bruni (1969). The slopes of the plots in Fig. 2 are determined by the magnitude of the dissociation constants of the enzyme–NADH complexes and, since these are within the experimental error the same, a dissociation constant of 10^{-5}M at 25°C in phosphate buffer, pH7.0 and I 0.1 (Dickinson, 1970), describes the binding of NADH to native and alkylated enzyme.

Although the dissociation constant of the enzyme–NADH complex was unaffected, it is clear from Fig. 2 that there was a loss of some 15% of the total coenzyme-binding capacity on inactivation with iodoacetamide. This effect may be explained. Apart

from the essential thiol groups there must be many groups in the enzyme capable of reaction and it seems probable that iodoacetamide may react with these other groups in a fairly random fashion. Thus, although all enzyme molecules must eventually have contained at least four substituted thiol groups, since all activity in the preparation was lost, some may have contained substantially more than eight. Some of the heavily substituted material may have been partly denatured and have lost coenzyme-binding capacity. Evidence to support this suggestion may be drawn from the fact that in the incubation mixtures with an eightfold excess of iodoacetamide, about 10% of the initial protein was lost as precipitate by the end of the 4h reaction period (see the Materials and Methods section). Presumably this small amount of protein became so heavily substituted that it was totally denatured.

The present results describing the interaction of NADH and alcohol dehydrogenase agree well with earlier work from this laboratory (Dickinson, 1970) but are at variance with the findings of Pfleiderer & Auricchio (1964) and Auricchio & Bruni (1969) who found as many as five NADH binding sites/molecule by using samples of enzyme obtained from a commercial source. In Fig. 2 are shown results obtained here in working with a preparation of enzyme from that source. The values obtained agree very well with those obtained by using home-produced enzyme, despite the fact that the commercial enzyme possessed only 60% of the activity of the fresh preparations. This latter observation also appears at variance with other results of Auricchio & Bruni (1969), which indicated that the specific activity of enzyme preparations was proportional to total coenzyme-binding capacity. An explanation for this discrepancy may possibly be found in the fact that even the most active preparations used by Auricchio & Bruni (1969) appear to have possessed only 30–50% of the activity of the preparations used here. The differences in total coenzyme-binding capacity noted above may be due to the different methods used for determination of protein. Pfleiderer & Auricchio (1964) and Auricchio & Bruni (1969) used a version of the biuret method and this was not apparently based on enzyme dry-weight determinations. In the present work enzyme concentrations were determined spectrophotometrically at 280nm by using a specific extinction coefficient $E_{1cm}^{1\%} = 12.6$, which is based on dry-weight measurements (Hayes & Velick, 1954; Dickinson, 1970).

The present results showing the similarity of coenzyme-binding capacity of enzyme preparations of differing specific activity is not necessarily surprising. The present work and that of Auricchio & Bruni (1969) shows that the blocking of certain thiol groups associated with enzymic activity does not affect coenzyme binding, whereas Bühner & Sund (1969) claim that the fall in specific activity of yeast alcohol
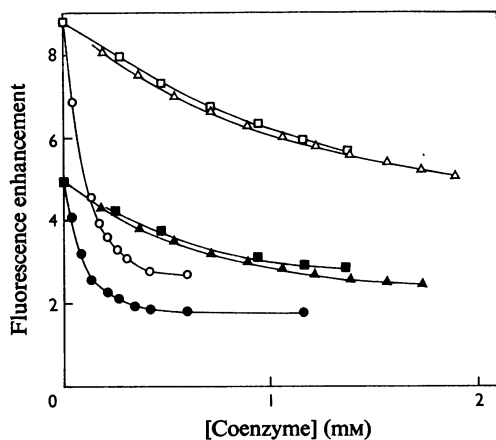
Fig. 3. *Effect of coenzyme on the fluorescence enhancement observed on mixing* 46 µM-*1-anilinonaphthalene-8-sulphonate and* 43 µM *native or inactivated yeast alcohol dehydrogenase*

Experimental details are given in the text. For titrations with native enzyme: ●, NADH; ▲, NAD⁺; ■, PAAD⁺; for titrations with inactivated enzyme: ○, NADH; △, NAD⁺; □, PAAD⁺.

dehydrogenase preparations on storage is connected with the oxidation of thiol groups.

Fig. 3 shows the effect on the fluorescence enhancement observed on mixing native or inactivated alcohol dehydrogenase and 1-anilinonaphthalene-8-sulphonate when NAD^+, $PAAD^+$ or NADH were added to the mixtures. The changes in fluorescence enhancement with the native enzyme are thought to mirror changes in enzyme conformation after the binding of coenzyme or coenzyme analogue (Dickinson, 1971). Since similar changes occurred with the alkylated enzyme over the same concentration ranges it appears that the conformational changes occurring on coenzyme binding are unimpeded by reaction with iodoacetamide. In the case of NADH this is not surprising since it is established that the dissociation constant for the enzyme–NADH complex is not affected by alkylation of the essential thiol groups. The results with NAD^+ and $PAAD^+$ indicate that the binding of these compounds is similarly unaffected. Further, the results with NAD^+ and $PAAD^+$ suggest that the dissociation constants for these two compounds from binary complexes with the enzyme are very similar if not identical. Hayes & Velick (1954) estimated a value for the dissociation constant of the enzyme–NAD^+ complex of 2.6×10^{-4} M at 0°C in pyrophosphate–glycine buffer, pH 7.8. Values of about 3.5×10^{-4} M are suggested for the NAD^+ and $PAAD^+$ complexes from results shown in Fig. 3. In

the case of NAD^+ the value is quite consistent with that given by Hayes & Velick (1954) in view of the differences in experimental conditions. The results of Fig. 3 also yield an estimate for the dissociation constant of the enzyme–NADH complex of 1.5×10^{-5} M, in agreement with estimates from the more accurate method described earlier. It is noteworthy that the dissociation constants for NADH and reduced PAAD from binary complexes with the enzyme are also characterized by the same dissociation constant (Van Eys *et al.*, 1957).

It is apparent from Fig. 3 that there was a 1.7-fold increase in the fluorescence enhancement of enzyme and 1-anilinonaphthalene-8-sulphonate mixtures when the enzyme was inactivated with iodoacetamide. This change could have been due to an increase in the intrinsic fluorescence of bound 1-anilinonaphthalene-8-sulphonate or to an increased affinity of the inactivated enzyme for the dye. At present it is not possible to distinguish between these possibilities, neither is it clear why the change occurred. Enzyme treated with a twofold excess of iodoacetamide, and which had in consequence lost 50% of its initial activity (Fig. 1) by blockage of an average of two essential thiol residues per molecule, exhibited only one-quarter of the extra fluorescence observed for enzyme inactivated with an eightfold excess of iodoacetamide. Clearly alkylation of the essential thiol residues contributed to the change but no more, apparently, than other less vital groups in the enzyme.

The results so far described establish that the coenzyme-binding sites in alcohol dehydrogenase are fully preserved when the essential thiol groups are blocked by reaction with iodoacetamide. Further, the conformational changes in the enzyme which are associated with the binding of coenzyme are also unimpeded by alkylation. With both lactate dehydrogenase (Holbrook, 1966; Holbrook & Stinson, 1970) and glyceraldehyde 3-phosphate dehydrogenase (Friedrich, 1965) from pig heart and alcohol dehydrogenase from horse liver (Li & Vallee, 1965) coenzyme-binding capacity was retained despite the blocking of essential thiol groups. However, for the lactate dehydrogenase the dissociation constants of the enzyme–NADH complexes were increased three- to ten-fold depending on the blocking agent, whereas for the glyceraldehyde 3-phosphate dehydrogenase the apparent dissociation constant of the enzyme–NAD^+ complex was increased fourfold on inactivation with iodoacetate. For liver alcohol dehydrogenase the dissociation constant of the enzyme–NADH complex was increased almost 100-fold after inactivation with iodoacetate. The present case with yeast alcohol dehydrogenase is only unusual, apparently, in that the strength of coenzyme binding is unaffected by the blocking of the essential thiol groups.

It is plain that the role of the essential thiol groups

of alcohol dehydrogenase must be concerned with aspects of the catalytic mechanism other than those associated with coenzyme binding. There are alternative possibilities. The loss in activity on alkylation may be due to the loss of the ability to bind substrate to the enzyme–coenzyme complexes or to an interference with hydride transfer between substrate and coenzyme in the catalytic ternary complexes. The results below allow some discrimination between these alternatives.

Fig. 4 shows the results of fluorescence titration of native and inactivated alcohol dehydrogenase with NADH in the presence of 500mM-acetamide. As expected the titrations in the absence of acetamide show similar behaviour for both active and inactive enzymes. The increase in fluorescence intensity observed for the inactive enzyme in the absence of acetamide appears to be due to alkylation of the essential thiol groups. In 40μM-NADH, enzyme treated with a threefold excess of iodoacetamide gave three-quarters of the extra fluorescence intensity shown by enzyme treated with an eightfold excess. It seems that in the enzyme–NADH complex the essential thiol, when blocked by reaction with iodo-

acetamide, is sufficiently close to the reduced nicotinamide moiety to cause some small perturbation of the fluorescence properties of that group. However, this interaction is clearly not of sufficient strength to cause any detectable change in the dissociation constant of the enzyme–NADH complex.

The results of the fluorescence titrations conducted in the presence of acetamide shown in Fig. 4 indicate a very clear difference between native and inactivated enzyme. The large enhancement in fluorescence intensity observed for the untreated enzyme was due to the formation of an enzyme–NADH–acetamide complex, which has a relatively high intrinsic fluorescence (Dickinson, 1970). The complete absence of this increased fluorescence with alkylated enzyme indicates that it was unable to form the ternary complex.

Fig. 5 shows the changes in extinction at 315nm after the incubation at 4°C of native and inactivated alcohol dehydrogenase with PAAD$^+$ in 20mM-hydroxylamine. The changes observed with the native enzyme were due to the formation of an enzyme–PAAD$^+$–hydroxylamine complex (Van Eys et al., 1957; Dickinson, 1970). Again the inactivated enzyme showed no evidence for the formation of the ternary complex. The inactivated enzyme was quite capable of binding PAAD$^+$ as evidenced by the fluorescence changes shown in Fig. 3.

It seems possible that the ternary complexes discussed above are analogous to the ternary complexes of enzyme, coenzyme and substrate formed in catalysis. If this is accepted the results described indicate
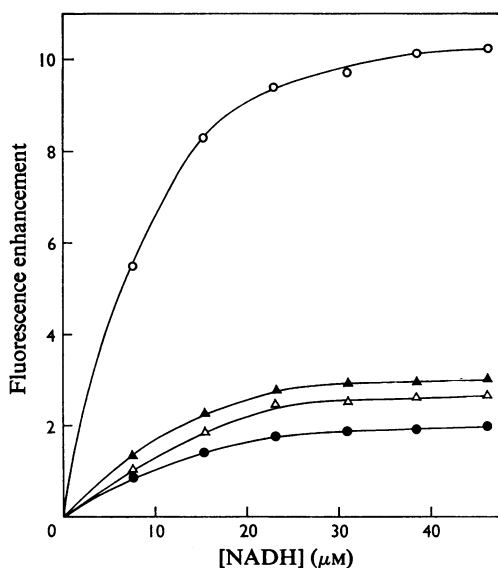

Fig. 4. *Fluorescence titrations at 25°C of 5 μM native or inactivated yeast alcohol dehydrogenase with NADH in the presence and in the absence of 500mM-acetamide*

Experimental details are given in the text. ●, Native enzyme+NADH; ▲, inactivated enzyme+NADH; ○, native enzyme+NADH+acetamide; △, inactivated enzyme+NADH+acetamide.
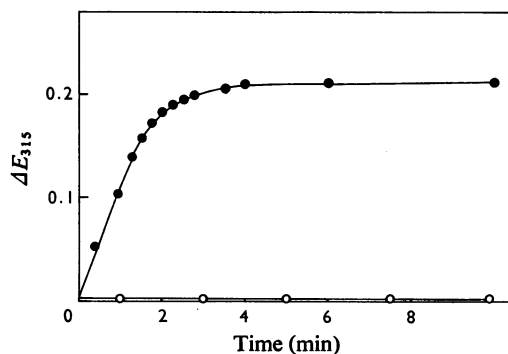

Fig. 5. *Time-course for the formation of yeast alcohol dehydrogenase-PAAD$^+$-hydroxylamine complex at pH7.0 and 4°C in mixtures of 330μM-PAAD$^+$, 20mM-hydroxylamine and 11 μM native or inactivated enzyme*

Experimental details are given in the text. ●, Native enzyme+PAAD$^+$+hydroxylamine; ○, inactivated enzyme+PAAD$^+$+hydroxylamine.

that loss of enzymic activity on alkylation with iodo-acetamide results from a failure of substrate to bind properly to enzyme–coenzyme complexes which involve inactivated enzyme. It may be argued that ternary complexes enzyme–NADH–acetamide and enzyme–PAAD⁺–hydroxylamine are formed with inactivated enzyme but that the changes in spectral properties characteristic of the native enzyme are lost. At present this argument cannot be set aside, but in any case it is obvious that the normal ternary complexes are not formed. With pig heart lactate dehydrogenase (Holbrook & Stinson, 1970) modification of the essential thiol groups has been shown to produce a loss of the ability to form the abortive enzyme–NAD⁺–pyruvate complex and of the ability to form the product complex enzyme–NADH–oxalate. In contrast, horse liver alcohol dehydrogenase apparently retains the ability to form the abortive enzyme–NADH–ethanol complex despite the blocking of the essential thiol groups by iodo-acetate (Li & Vallee, 1965).

Rabin & Whitehead (1962) and Whitehead & Rabin (1964) have shown that the presence of NAD⁺ or NADH in incubation mixtures protects alcohol dehydrogenase from reaction with iodoacetamide. Their observations were consistent with a model in which coenzyme binding to the enzyme resulted in complete protection of the thiol group from reaction with the alkylating agent. Accordingly it was suggested (Rabin & Whitehead, 1962) that the thiol group is directly involved in coenzyme binding. This no longer appears to be correct and some alternative explanation for the protection of the thiol groups by coenzyme is required. For lactate dehydrogenase, protection from inactivation with maleimide by NADH has been explained on the basis of the thiol group being in a pocket whose entrance is closed on the binding of coenzyme (Holbrook & Stinson, 1970). The pocket is supposed to be deep enough to accommodate the modified group so that the binding of coenzyme is not seriously weakened in the inactivated enzyme. Such an explanation would accommodate many of the present results with alcohol dehydro-

genase but does not easily explain the loss of substrate-binding capacity by inactivated enzyme. An alternative hypothesis is suggested by the experiments with 1-anilinonaphthalene-8-sulphonate. The conformational change taking place on the binding of coenzyme may result in the movement of the thiol group to a position close to the coenzyme. In the new situation the thiol group may be protected from attack by iodoacetamide or if already alkylated the blocked group may interfere with the approach of substrate to the bound coenzyme. The perturbation of the fluorescence of NADH bound by the inactivated enzyme (Fig. 4) suggests that with native enzyme the thiol groups may be close to the reduced nicotinamide moiety of bound NADH.

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