THE ROLE OF THE —SH GROUPS OF YEAST ALCOHOL DEHYDROGENASE IN THE OXIDATION OF DIFFERENT SUBSTRATES

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It has been known for long that yeast ADH (alcohol dehydrogenase) is a —SH enzyme and the blocking of the —SH groups with monoiodoacetic acid, PCMB (p-chloromercuribenzoate), salyrgan and other mercaptide-forming compounds or their oxidation inhibits the activity of the enzyme [1—11].

It has been shown that the yeast ADH is a groupspecific enzyme capable of oxidizing not only ethanol, but also a variety of other alcohols [5, 12—18].

As it is also known that

1. certain groupspecific enzymes, as for instance the mammalian muscle PGAD (D-glyceraldehyde-3-phosphate dehydrogenase) utilize different active —SH groups in the oxidation of different aldehyd substrates [19], and

2. the —SH groups of the baker's yeast ADH are not involved in the H-transfer and are required only as coenzyme-binding groups [20—28]

in the present work we subjected to study the role of the —SH groups of yeast ADH in the oxidation of different substrates.

As there are substantial differences between the crystalline ADHs isolated from baker's and brewer's yeast in regard to structure [8—10], kinetic [8—10, 29, 30] and immunological properties [31, 32], the role of the —SH groups has been investigated in the case of both kinds of ADH.

Methods

The crystalline ADH used in the experiments had been isolated from baker's yeast and from brewer's yeast, partly by RACKER's [33], partly by KELETI's [9, 34] methods.

PCMB : This was a 88.5 per cent preparation analysed for Hg content and by the optical method [35], in an *N*/10 glycine buffer (pH 8.4) solution.

The different alcohols were purified by distillation.

DPN : This was a 85 per cent *Light* preparation.

Enzymatic activity was measured as described earlier [9], except that the *N*/10 glycine buffer used contained no KCN. The pH of the buffer was 8.4.

The number of —SH groups was determined by a spectrophotometrical method [35].

The oxidation of alcohol in the presence of alloxane was determined by the optical test [28].

The spectrophotometer used was of the Hilger *UVISPEK* type. The measurements were made at room temperature, in silica cells of 1 cm pathlight.

Results

The ADH preparations used in the experiments had been tested for the number of —SH groups they contained. It was found that both kinds of ADH contained 36 to 40 mols of free —SH groups per mol protein titratable with PCMB (Fig. 1).

The method by which the crystalline protein is prepared has no influence on the number of detectable —SH groups.

We have investigated the oxidation of different substrates in the presence of the two kinds of yeast ADH. It has been found that in the presence of brewer's yeast ADH the relative rate of oxidation of straight C-chain alcohols decreased

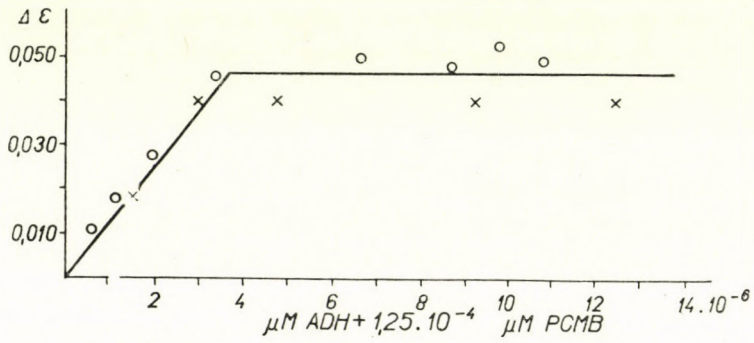

Fig. 1. Determination of the number of —SH groups in yeast ADH
○ : baker's yeast ADH × : brewer's yeast ADH
The enzyme had been preincubated with PCMB at room temperature for 25 to 40 minutes. The measurements were made at 255 mμ

exponentially with the increase in the number of C atoms. In agreement with data in the literature [18] identical results were obtained for the baker's yeast ADH (Fig. 2).

In studies on the oxidation of alcohols with branched C-chain it has been found that in the case of the baker's yeast ADH the relative rate of reaction decreased linearly with the increase in the number of the C atoms in the main chain. When the brewer's yeast ADH was used, no differences were found on grounds of the number of the C atoms in the main chain and the enzyme oxidized the alcohols with branched C-chain at an equal rate (Fig. 3).

In accordance with the data in the literature [18], benzylalcohol and lactic acid (without ADP) proved to be very poor substrates for the baker's yeast ADH. We have shown that the brewer's yeast ADH was even less active than the baker's yeast ADH on benzylalcohol and lactic acid (without ADP). The relative rate of oxidation of benzylalcohol (as related to the rate of oxidation of ethanol) by brewer's yeast ADH was but 50 per cent of the value measured

in the case of baker's yeast ADH; in the case of lactic acid oxidation the value obtained was 1/4 of these obtained with baker's yeast ADH.

By blocking the —SH groups of the two kinds of ADH it was found that it sufficed to block 2 to 3 —SH groups with PCMB (in accordance with the earlier data obtained for the oxidation of ethanol [9, 10]) to inhibit the oxidation of

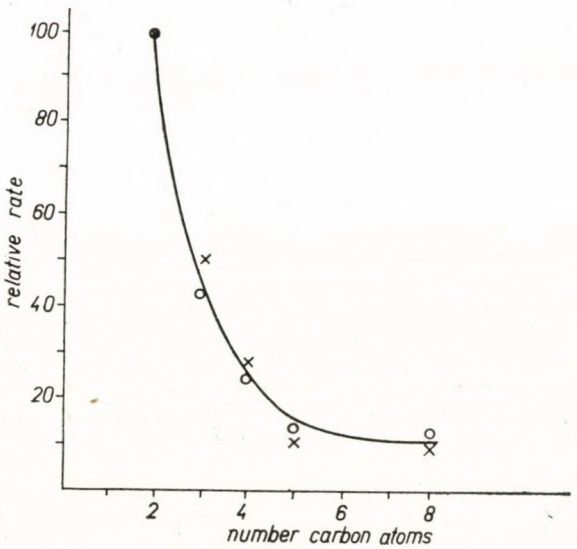

Fig. 2. Effect of the increase in the length of the C-chain on the rate of oxidation of straight C-chain alcohols

○ : baker's yeast ADH × : brewer's yeast ADH
Reaction mixture : pH 8.4 glycine buffer (0.033 *M*), DPN ($2.5 \cdot 10^{-3} M$), alcohol (0.3 *M*), ADH (10 μg/ml), in end concentrations
The rate of ethanol oxidation is taken arbitrarily to be 100, and all the other values are related to this

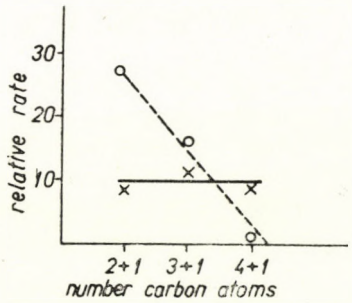

Fig. 3. Effect of the increase in the length of the C-chain on the rate of oxidation of branched C-chain alcohols

○ : baker's yeast ADH × : brewer's yeast ADH
Reaction mixture : pH 8.4 glycine buffer (0.033 *M*), DPN ($2.5 \cdot 10^{-3} M$), alcohol (0.3 *M*), ADH (20 μg/ml), in end concentrations
Values related to the value of ethanol oxidation, taken arbitrarily to represent 100

straight C-chain alcohols. On the other hand, about 40 equivalent PCMB could inhibit in 100 per cent the oxidation of alcohols with branched C-chain (Fig. 4).

When alloxane was used as the coenzyme, instead of DPN [28] the baker's yeast ADH could oxidize only the straight C-chain alcohols and could not use the alcohols with branched C-chain as substrate under such conditions.

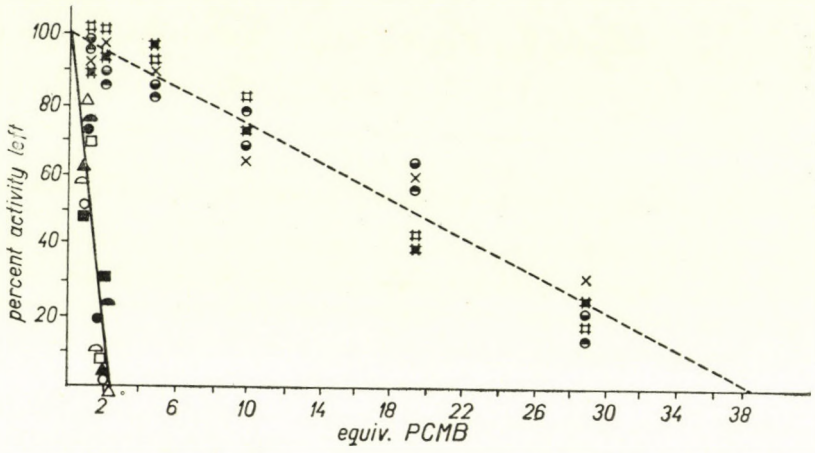

Fig. 4. Effect of the blocking — SH groups on the oxidation of different alcohols

○ : baker's yeast ADH with ethanol substrate
△ : ,, ,, ,, ,, propylalcohol ,,
□ : ,, ,, ,, ,, butylalcohol ,,
⌓ : ,, ,, ,, ,, amylalcohol ,,
● : brewer's yeast ADH with ethanol substrate
▲ : ,, ,, ,, ,, propylalcohol ,,
■ : ,, ,, ,, ,, butylalcohol ,,
◗ : ,, ,, ,, ,, amylalcohol ,,
: baker's yeast ADH with isopropylalcohol substrate
◔ : ,, ,, ,, ,, isobutylalcohol ,,
#● : brewer's yeast ADH with isopropylalcohol substrate
◑ : ,, ,, ,, ,, isobutylalcohol ,,
× : ,, ,, ,, ,, isoamylalcohol ,,

The enzyme had been preincubated with PCMB for 25 to 40 minutes at room temperature. Reaction mixture: as in *Fig. 3*

In the presence of DPN $5 \cdot 10^{-2}$ *M* alloxane inhibits the oxidation of ethanol [8—10]. According to the present investigations it inhibited at about the same extent the oxidation of the other straight C-chain alcohols, whereas it completely blocked the enzyme reaction taking place in the presence of alcohols with branched C-chain.

On the other hand, when alloxane was used in a concentration of $1.4 \cdot 10^{-3} M$ an activating effect could be observed in the enzyme reaction taking place in the presence of DPN. The oxidation rate of ethanol with DPN, in the presence of $1.4 \cdot 10^{-3}$ *M* alloxane does not differ from that measured in the absence of alloxane. However, in the case of the other substrates (both the straight and the

branched C-chain alcohols) the oxidation rate was substantially higher than in the absence of alloxane. The results are presented in Table I.

Table I

Alcohol oxidation in the presence of DPN and of DPN + $1.4 \cdot 10^{-3}$ M *alloxane, respectively*

Alcohol tested	Activity of the enzyme in presence of	
	DPN	DPN + $+ 1.4 \cdot 10^{-3}$ *M* alloxane
Ethanol	100*	100
Propylalcohol	44	83
Butylalcohol	26	61
Amylalcohol	14	30
Isopropylalcohol	27	91
Isobutylalcohol	17	44
Isoamylalcohol	0.2	21

* Arbitrary value. All the other values are related to this. Reaction mixture: see in *Fig. 3*. Baker's yeast ADH

Discussion

Experimental evidence has been obtained showing that there was no difference in the number of free —SH groups between the baker's yeast and the brewer's yeast ADHs.

According to the data published by VAN EYS, CIOTTI and KAPLAN [21], the baker's yeast ADH has 18.5 —SH groups per mol, whereas BARRON and LEVINE [5] found 20 and WALLENFELS and SUND [7, 11] 36 —SH groups. The latter showed that 36 free —SH groups can be found only in the presence of maximal enzyme activity (turnover number 28 600). As the enzyme is being inactivated, the number of the free —SH groups decreases parallel with the decrease in activity. This is suggested to explain the fact that other authors have found a smaller number of free —SH groups in the ADH. According to their data, there is a linear correlation between the number of free —SH groups and enzyme activity. This statement seems to be valid only up to a certain limit (to the turnover number 28 600), as we have found the same number of —SH groups (36 to 40 mol per mol protein) in the case of our enzyme preparations [34], that had a turnover number almost the double of that mentioned above. It appears that in this case the increase in enzyme activity resulted not from a liberation of further —SH groups, but from the formation of complexes with the traces of heavy metals interfering with the action of Zn atoms required for enzyme activity [36—39, 8—10, 28, 34].

The two kinds of yeast ADH showed no differences in the oxidation of various alcohols with straight C-chain. However, a difference could be demonstrated in the oxidation of the different alcohols with branched C-chains. The baker's yeast ADH oxidizes these substrates in a measure decreasing linearly with the increase in the number of C atoms, whereas the brewer's yeast ADH oxidizes at an equal rate the alcohols with branched C-chain we have tested. In addition to the numerous structural [8—10], kinetical [8—10, 30] and immunological [31, 32] differences, this is the first *functional* difference demonstrated to exist between the two enzymes isolated from closely related species.

On blocking the —SH groups of baker's yeast ADH with different amounts of PCMB it was found that it sufficed to block 2 to 3 —SH groups to inhibit the oxidation of straight C-chain alcohols. In contrast with this, all the —SH groups of ADH should be blocked to inhibit the oxidation of alcohols with branched C-chain. Similar results were obtained in the experiments with brewer's yeast ADH. This indicates that the same number of —SH groups is responsible for the oxidation of branched C-chain alcohols in both kinds of ADH.

We have found that alloxane has a coenzyme-like action only in the case of the oxidation of straight C-chain alcohols, but fails to participate in the oxidation of alcohols with branched C-chain. $5 \cdot 10^{-2} M$ alloxan inhibits completely the oxidation of branched C-chain alcohols in the presence of DPN.

The experimental results, according to which in the presence of DPN $1.4 \cdot 10^{-3} M$ alloxane activates the oxidation of all the straight and branched C-chain alcohols we tested (with the exception of ethanol) suggest that in this case, the binding of certain coenzyme-binding groups may have an activating effect on the enzyme molecule, as a whole.

These results and those reported in the literature make it possible to draw some conclusions as to the active centres in the yeast ADH. HAYES and VELICK [29] have shown baker's yeast to possess four active centres, which are absolutely equivalent. Similar statements have been made by KAPLAN *et al.* [21, 22]. In studies on the inhibition of ADH activity with its specific antiserum, ANTONI and KELETI [31,32] have found that the antigen — antibody ratio required for the complete inhibition of enzyme activity is 1 : 4 (the molecular weight of γ-globulin and of ADH being equally 150 000). This lends support to the above view concerning the equivalence of the active centres. At the same time, however, KAPLAN *et al.* [21, 22] stated that only one coenzyme molecule is reduced at a time. They also showed [18] that only one alcohol molecule is attached to the enzyme molecule at a time. If one of the 4 DPN molecules attached to the enzyme is reduced to DPNH, lactic acid and propylalcohol are oxidized at a higher rate [18].

These results may suggest that the active centres of ADH are not equivalent after all. However, the above results are required, but not the sufficient criteria of the above suggestions. It has been also demonstrated that, using

alloxane a concentration of $1.4 \cdot 10^{-3}$ *M* suffices to attain coenzyme saturation in the ethanol oxidation reaction of yeast ADH. Under such conditions the enzyme shows 1/4 of the activity attainable in the presence of DPN [28]. However, in the presence of $5 \cdot 10^{-2}$ *M* alloxane, ADH shows the same activity as in the presence of DPN as coenzyme [9, 10].

In spite of the fact that there are 8 —SH groups participating in the binding of the coenzyme in the 4 active centres [21, 22], 2 to 3 equivalent PCMB can inhibit completely the oxidation of straight C-chain alcohols. And this is about corresponding to the number of —SH groups in a single active centre. All these results can be explained only if it is assumed that one of the four active groups in ADH is not equivalent with the other three, but contains more reactive SH groups. This suggestion would fully explain the phenomena described by KAPLAN *et al.* [18, 21, 22], too.

Thus, the yeast ADH may contain three kinds of active centre :

1. *Primary centre.* It contains 2 —SH groups and 1 Zn atom. Its inhibition blocks the oxidation of straight C-chain alcohols. It combines readily with alloxane. The binding of DPNH to this centre has an activating effect on the reactions catalysed by the enzyme, just as if this centre is bound by alloxane.

2. *Secondary centres.* They contain 6 —SH groups and 3 Zn atoms. They participate in the oxidation of straight C-chain alcohols. They can combine with alloxane, but only in the presence of high concentrations. From the point of view of immunological inhibition they do not differ in behaviour from the primary centre. The reduction of DPN bound to these centres, or the formation of a linkage with alloxane does not activate the enzyme molecule.

3. *Tertiary centres.* These contain 28 to 32 —SH groups, but no Zn atoms. They take part in the oxidation of alcohols with branched C-chain. They cannot combine with alloxane and are inactivated under its influence when DPN is employed as a coenzyme.

SUMMARY

In the ADHs isolated from baker's yeast and from brewer's yeast 36 to 40 mols of free—SH groups per mol protein can be demonstrated.

The blocking of 2 to 3 —SH groups with PCMB inhibits the oxidation of straight C-chain alcohols, whereas all —SH groups should be blocked to inhibit the oxidation of alcohols with branched C-chain.

In the case of baker's yeast and brewer's yeast ADHs a similar correlation can be demonstrated between the number of C atoms in the different straight and the rate of oxidation of C-chain alcohols, these substrates, whereas in the case of alcohols with branched C-chain a functional difference is demonstrable between the two kinds of ADH.

When alloxane is used instead of DPN, both kinds of ADH can oxidize straight C-chain alcohols only. In the presence of DPN high concentrations of alloxane inhibit the oxidation of different alcohols, whereas small concentrations have an activating effect.

The role of the active centres of yeast ADH in the oxidation process of different alcohols is discussed.

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