Radloff, R., Bauer, W., and Vinograd, J. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 1514.
Rickwood, D. (1972), *Biochim. Biophys. Acta 269,* 47.
Robberson, D., Aloni, Y., Attardi, G., and Davidson, N. (1971), *J. Mol. Biol. 60,* 473.
Robberson, D. L., and Davidson, N. (1972), *Biochemistry 11,* 533.
Saxinger, W. C., Ponnamperuma, C., and Gillespie, D. (1972), *Proc. Nat. Acad. Sci. U. S. 69,* 2975.
Shih, T. Y., and Martin, M. A. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1697.
Smith, I., Smith, H., and Pifko, S. (1972), *Anal. Biochem. 48,* 27.
Sullivan, N., and Roberts, W. K. (1973), *Biochemistry 12,* 2395.
Tibbetts, C., Johansson, K., and Philipson, L. (1973), *J. Virol. 12,* 218.
Wagner, A. F., Bugiannesi, R. L., and Shen, T. Y. (1971), *Biochem. Biophys. Res. Commun. 45,* 184.
Westphal, H. (1970), *J. Mol. Biol. 50,* 407.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31,* 349.
Yoshiike, K. (1968), *Virology 34,* 391.

Effect of pH on the Reactivity of the Active-Site Sulfhydryl Groups in Yeast Alcohol Dehydrogenase†

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ABSTRACT: The relative reactivity of the two active-site sulfhydryl groups (designated X and Y) in yeast alcohol dehydrogenase is determined by both the nature of the reagent used and the pH at which the reaction is carried out. Iodoacetate modifies 30% of the X-sulfhydryl and 70% of the Y-sulfhydryl at pH 5.7, but is specific for the Y-sulfhydryl at pH 7.5. For the uncharged reagents butyl isocyanate and iodoacetamide the pH effect is less pronounced. The former reagent modifies 55–60% of the X-sulfhydryl and 40–45% of the Y-sulfhydryl at both pH 5.7 and 6.5, and the latter reagent modifies about 40% of the X-sulfhydryl and 60% of the Y-sulfhydryl at pH 5.7 and 6.5 and 25% of X-sulfhydryl and 75% of the Y-sulfhydryl at pH 7.5. The Y-sulfhydryl has now been identified as Cys-43 in the primary sequence of yeast alcohol dehydrogenase (Jörnvall, H. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2295), but the location of the X-sulfhydryl is still unknown.

Evidence has been presented in previous publications that yeast alcohol dehydrogenase contains two distinct active-site sulfhydryl groups, both of which appear to be required for catalytic activity. One of the sulfhydryl groups (designated X) reacts preferentially with butyl isocyanate at pH 5.7 (Twu and Wold, 1973), and the other (designated Y) reacts exclusively with iodoacetate at pH 7.5 (Harris, 1964). Both sulfhydryl groups react with iodoacetamide at pH 6.5 (Twu *et al.*, 1973). With all three reagents an inactive enzyme derivative is obtained when one sulfhydryl per active site has been modified, and it can be demonstrated that the specific reactivity of either one sulfhydryl group is lost when the other reacts. It was recognized in a previous report (Twu *et al.*, 1973) that the observed difference in the reactivity of the two sulfhydryl groups could reflect either subtle differences in the reaction specificity of iodoacetate, iodoacetamide, and butyl isocyanate, or effects of pH on the relative reactivity of the two sulfhydryl groups toward all three reagents. The purpose of the work reported in the present paper was to try to distinguish between these two possibilities.

Experimental Section

Materials. Yeast alcohol dehydrogenase (crystallized and lyophilized; 400 units/mg) was purchased from Sigma Chemical Co. and was of the same quality as the preparations used in previous work in our laboratory (Twu and Wold, 1973; Twu *et al.*, 1973). [^{14}C]Butyl isocyanate (labeled in the carbonyl carbon, 3.25 Ci/mol), [1-^{14}C]iodoacetamide (12.2 Ci/mol), [1-^{14}C]iodoacetic acid (13.45 Ci/mol), and [^{3}H]iodoacetic acid (67.2 and 357 Ci/mol) were obtained from New England Nuclear. If the radioactive samples were diluted before use, the specific radioactivity of the final reagent sample was determined by preparing the corresponding *S*-cysteine derivative and relating the radioactive counts directly to micromolar concentration of derivative as determined on the long column of the amino acid analyzer.

Assays and Methods. The procedures for enzyme assays, protein assays, and amino acid analyses, as well as the determination of radioactivity, have been described in a previous paper (Twu and Wold, 1973). The modification of dehydrogenase with butyl isocyanate, iodoacetate, and iodoacetamide also followed previously described methods (Twu and Wold, 1973; Twu *et al.*, 1973; Whitehead and Rabin, 1964; Rabin *et al.*, 1964). In our hands, the specificity of the inactivation with the alkylating reagents decreased significantly at low pH, and complete inactivation was often associated with incorporation of more than the expected 3–4 mol of reagent/mol of protein.

Results

To establish whether the two different sulfhydryl groups react to a different extent at different pH, it was first required to ascertain that the same two sulfhydryl groups are involved in the reaction over the whole pH range. To this end two samples of 100 mg each of yeast alcohol dehydrogenase in 20 ml of 0.1 M potassium phosphate buffer, one at pH 5.7 and one at pH 7.5, were treated in parallel with 7 mol/mol of enzyme of [^{14}C]iodoacetate and [^{3}H]iodoacetate, respectively. The course of the inactivation was checked carefully to avoid overreaction. When the activity had dropped to below 10%, the samples were subjected to gel filtration to remove excess reagent and after

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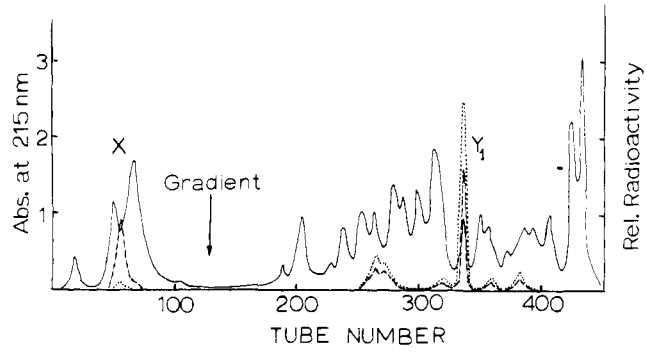

FIGURE 1: The elution pattern from phosphocellulose of the peptic peptides from a mixture of yeast alcohol dehydrogenase inactivated with [^{14}C]iodoacetate at pH 5.7 and with [^{3}H]iodoacetate at pH 7.5. The solid line trace represents absorbance at 215 nm, the broken line ^{14}C counts, and the dotted line ^{3}H counts. The peptide mixture (80 mg) in 0.02 N H_3PO_4 was applied to a 1.5 × 40 cm column of phosphocellulose equilibrated with 0.025 N H_3PO_4. The column was eluted with 0.025 N H_3PO_4 through fraction 120, then with a three-chamber gradient of 350 ml each of 0.025 N H_3PO_4, 0.01 M KCl in 0.025 N H_3PO_4, and 0.2 M KCl in 0.025 N H_3PO_4 through fraction 410, and finally with 0.5 M KCl in 0.025 N H_3PO_4. The two major radioactive peptides are designated X and Y_1.

TABLE I: Effect of pH on the Relative Reactivity of the Two Sulfhydryl Groups of Yeast Alcohol Dehydrogenase; Per Cent Distribution of Recovered Radioactivity in the Early (X) Peptide and the Late (Y) Peptide Fractions.

Reagent (Deg of Incorpn)	Fraction[a]	pH 5.7	pH 6.5	pH 7.5
[^{14}C]Butyl isocyanate (3.7 mol/mol)	X	59	55	
	Y_1	24	12	
	Y_2	17	33	
[^{14}C]Iodoacetamide (1.7–3.0 mol/mol)	X	42	42	24
	Y_1	21	12	40
	Y_2	37	46	36
[^{3}H]Iodoacetate (0.8–1.5 mol/mol)	X	35	19	7
	Y_1	23	42	59
	Y_2	42	39	34
Iodoacetate (from expt in Figure 1) (1.9–3.0 mol/mol)	X	27		1
	Y_1	45		62
	Y_2	28		37

[a] Fraction Y_1 represents the major late peak (at tube 340 in Figure 1) and Y_2 the sum of the minor peaks observed between tubes 250 and 400.

removing small aliquots of the resulting protein derivatives for characterization, the two derivatives were mixed, lyophilized, and subjected to pepsin digestion in a single incubation. When the digest was subjected to chromatography on phosphocellulose (Figure 1), the ^{14}C and ^{3}H labels eluted in identical positions, showing that there are no unique sulfhydryl groups reacting at different pH values. The ratio of the two labels in the peaks identified as representing the active-site sulfhydryl peptides differed markedly, however. The early fraction corresponding to the X-sulfhydryl contained 27% of the total ^{14}C label (inactivation at pH 5.7) but only about 1% of the ^{3}H label (inactivation at pH 7.5). The retarded peak corresponding to the Y-sulfhydryl, on the other hand, contained 45% of the ^{14}C and 62% of the ^{3}H label. In addition to these two major peaks there were five minor radioactive peaks accounting for a total of 28% of the ^{14}C and 37% of the ^{3}H (Figure 1). Since these minor peaks elute close to the major Y-peptide and since their isotope ratios are characteristic of that of the major Y-peptide we conclude that the minor fractions correspond to different segments of the main Y-peptide arising from different peptic cleavages. This experiment clearly demonstrates that the same two sulfhydryl groups react at pH 5.7 and 7.5, and that the X-sulfhydryl is modified to a significant extent by iodoacetate only at the low pH.

The amino acid composition of the two major peptides was found to correspond with the sequences established for the two sulfhydryl peptides obtained after reaction with butyl isocyanate and iodoacetamide (Twu and Wold, 1973; Twu *et al.*, 1973), and this confirmation of the reproducible elution position of modified peptides X and Y from the phosphocellulose column provided the basis for the main experimental test of the pH effect on the reactivity of the two sulfhydryl groups.

Three samples of yeast alcohol dehydrogenase were prepared in 0.1 M potassium phosphate buffer at pH 5.7, 6.5, and 7.5, respectively, all at a concentration of 5 mg/ml. The samples were divided into 5-ml (25 mg of enzyme) samples and each sample was treated with either [^{14}C]butyl isocyanate (at pH 5.7 and 6.5 only, since this reagent does not yield a stable derivative with sulfhydryl groups above pH 7 (Twu and Wold, 1973)), with [^{14}C]iodoacetamide (pH 5.7, 6.5, and 7.5), or with [^{3}H]iodoacetate (pH 5.7, 6.5, and 7.5). To ensure against nonspecific overreactions, the amounts of reagent added were adjusted to give less than stoichiometric reagent incorporation. The samples were incubated at room temperature for 1 hr for the carbamoylation reactions and the carboxymethylation reactions and for 3 hr for the carboxamidomethylation reactions; the reactions were then stopped by separating the enzyme from excess reagent. Three of the reaction mixtures, the iodoacetamide reactions at pH 5.7 and 6.5 and the iodoacetate reaction at pH 5.7, contained a good deal of precipitated protein, and these samples were treated with ice-cold acid acetone (acetone containing 10 ml of 0.1 M HCl/l.). The protein from each sample was precipitated and washed until no more radioactivity was obtained in the acetone washes. The other homogeneous reaction mixtures were gel filtered on Sephadex G-25, and the resulting protein fractions were pooled and lyophilized. Samples were removed for the determination of total reagent incorporation, and the rest of each derivative was dissolved or suspended in 0.02 N H_3PO_4 and subjected to pepsin digestion (Twu and Wold, 1973). Each of the resulting peptide mixtures was finally fractionated by ion exchange chromatography on phosphocellulose under the conditions described in Figure 1, and the distribution of radioactivity in the X region and the Y region (see Figure 1) was scored for each sample. The results are summarized in Table I.

In evaluating these results it must be emphasized that as shown in Figure 1 the Y region was always made up of several peaks, one major one eluting about in the middle of the KCl gradient, and four–five smaller ones on either side of the main peak. Based solely on the constant ^{14}C/^{3}H ratio observed for all these peaks in Figure 1, we have assumed that they represent the same sulfhydryl peptide, and arise because of heterogeneous pepsin cleavages. The major peak is recorded as Y_1 in Table I; the minor ones are added together and recorded as Y_2. The X fraction, eluting early at about 3 column volumes, was a single peak in all cases. It should also be noted that as expected the carboxymethyl peptides eluted from the column in a position slightly but consistently ahead of the position at which both the butylcarbamoyl peptides and the carboxamidomethyl peptides were observed.

The results in Table I show that the relative reactivity of the

two active-site sulfhydryl groups in yeast alcohol dehydrogenase is determined by both the nature of the reagent used and by the pH at which the reaction is carried out. The pH effect is best illustrated by the iodoacetate reaction which goes from about 30% reaction at the X position at pH 5.7 to essentially no reaction at pH 7.5. This is consistent with the observation of Harris (1964) that a single sulfhydryl group (the Y-sulfhydryl) is modified by iodoacetate at pH 7.5. In the case of the two uncharged reagents the X-sulfhydryl is relatively more reactive and the effect of pH is less pronounced, although in going from pH 6.5 to 7.5 the carboxamidomethylation definitely shifts from the X-sulfhydryl toward the Y-sulfhydryl. From the obvious difference in the distribution of the three reagents between the two sulfhydryl groups at any pH we conclude that the three reagents, although they all behave as active-site specific reagents, are positioned in different orientations in the active site. From the pH effect, we further conclude that an ionizable group in the active site affects either reagent orientation or sulfhydryl reactivity or both. It is possible that the ionizing group could be one of the two critical sulfhydryl groups, but other groups, such as a histidine residue, are also likely possibilities.

Discussion

The results presented here confirm the existence of two critical sulfhydryl groups in or near the active site of yeast alcohol dehydrogenase. Although the distinction made in this work is based on elution position only, it must be recalled that the X-peptide and the Y-peptide have been characterized and shown to be readily distinguishable. The X-peptide, eluting early from the phosphocellulose column, contains no basic residues and was determined in the studies of the butylcarbamoylated enzyme to be a hexapeptide with the sequence Cys-Ala-Gly-Ile-Thr-Val (Twu and Wold, 1973). The carboxamidomethylated X-peptide was subsequently shown to be the same one (Twu *et al.*, 1973) and the carboxymethylated X-peptide obtained from the chromatogram shown in Figure 1, after purification, gave an amino acid analysis consistent with this sequence as well. The Y-peptide, retarded on the phosphocellulose column and containing basic amino acids, was concluded to consist of a sequence overlapping the tryptic peptide characterized by Harris (1964). Based on the amino acid analysis of the carboxamidomethylated Y-peptide (Twu *et al.*, 1973), confirmed completely by the analysis of the carboxymethyl Y-peptide in this work, we assigned the sequence (Asp,Ile,Val)-Lys-Tyr-Ser-Gly-Val-Cys-His-Thr-Asp-Leu to this peptide, using Harris' (1964) tryptic peptide sequence and allowing for the expected differences in tryptic and peptic cleavages. Jörnvall (1973) has recently reported the complete sequence of the amino-terminal half of the yeast alcohol dehydrogenase subunit, and it is now consequently possible to identify the Y-sulfhydryl specifically in this known sequence. The correct sequence of the peptic peptide isolated in our laboratory should be Leu-Ile-Asn-Val-Lys-Tyr-Ser-Gly-Val-Cys-His-Thr-Asp, corresponding to residues 34–46 of yeast alcohol dehydrogenase. The only ambiguity left in this assignment is whether the leucine residue is at the amino-terminal end or at the carboxy-terminal end (residue 47 is also leucine). Because of high probability of a peptic cleavage at the Asp-Leu bond, we favor the above assignment.

The hexapeptide containing the X-sulfhydryl is not present in the amino-terminal half of the molecule, and further work must be done before the location of this second active-site residue can be located in the primary sequence of yeast alcohol dehydrogenase.

References

Harris, J. I. (1964), *in* Structure and Activity of Enzymes, Goodwin, T. W., Harris, J. I., and Hartley, B. S., Ed., New York, N. Y., Academic Press, p 97.

Jörnvall, H. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 2295.

Rabin, R. R., Cruz, J. R., Watts, D. C., and Whitehead, E. P. (1964), *Biochem. J. 90,* 539.

Twu, J. S., Chin, C. C. Q., and Wold, F. (1973), *Biochemistry 12,* 2856.

Twu, J. S., and Wold, F. (1973), *Biochemistry 12,* 381.

Whitehead, E. P., and Rabin, B. R. (1964), *Biochem. J. 90,* 532.

CORRECTION

"Studies on the Structure of Deoxyribonucleoproteins. Spectroscopic Characterization of the Ethidium Bromide Binding Sites," by Lynne M. Angerer, S. Georghiou, and E. N. Moudrianakis,* Volume 13, Number 6, March 12, 1974, page 1075.

Parentheses were incorrectly edited into eq 4 and 6 (p 1076); these equations should read:

$$c_b = \frac{\epsilon_f[(I/I_r)(c_r) - c_0]}{(\epsilon_b q_b/q_f) - \epsilon_f} \quad (4)$$

$$c_b = \frac{(I/I_r)(c_r) - c_0}{R - 1} \quad (6)$$

It is obvious that eq 1–3 could never yield eq 4 and 6 as originally printed. The results and conclusions reported in this paper are based on eq 4 and 6 in their corrected forms.