Hermann, R., Rudolph, R., & Jaenicke, R. (1979) *Nature (London) 277*, 243-245.
Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry* (in press).
Jaenicke, R. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., Ed.) pp 71-90, Springer-Verlag, Berlin, Heidelberg, and New York.
Jaenicke, R. (1971) *Eur. J. Biochem. 21*, 110-115.
Jaenicke, R. (1974) *Eur. J. Biochem. 46*, 149-155.
Jaenicke, R. (1978) *Naturwissenschaften 65*, 569-577.
Jaenicke, R. (1979) *FEBS-Symp. 52*, 182-198.
Jaenicke, R., & Koberstein, R. (1971) *FEBS Lett. 17*, 351-354.
Jaenicke, R., & Rudolph, R. (1977) *FEBS-Symp. 49*, 351-367.
Jaenicke, R., & Rudolph, R. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 525-548, Elsevier/North-Holland, Amsterdam.
Jaenicke, R., Koberstein, R., & Teuscher, B. (1971) *Eur. J. Biochem. 23*, 150-159.
Jaenicke, R., Engelhard, M., Kraus, E., & Rudolph, R. (1975) *Biochem. Soc. Trans. 3*, 1051-1054.
Joly, M. (1965) *A Physico-chemical Approach to Protein Denaturation*, Academic Press, New York.
Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1-63.
Levi, A. S., & Kaplan, N. O. (1971) *J. Biol. Chem. 246*, 6409-6417.
Levitzki, A. (1972) *FEBS Lett. 24*, 301-304.
Lin, L.-N., & Brandts, J. F. (1978) *Biochemistry 17*, 4102-4110.
Müller, K., Lüdemann, H.-D., & Jaenicke, R. (1981) *Biophys. Chem.* (in press).
Rudolph, R., Heider, I., Westhof, E., & Jaenicke, R. (1977a) *Biochemistry 16*, 3384-3390.
Rudolph, R., Heider, I., & Jaenicke, R. (1977b) *Biochemistry 16*, 5527-5531.
Rudolph, R., Zettlmeissl, G., & Jaenicke, R. (1979) *Biochemistry 18*, 5572-5575.
Schade, B. C., Lüdemann, H.-D., Rudolph, R., & Jaenicke, R. (1980a) *Biophys. Chem. 11*, 257-263.
Schade, B. C., Rudolph, R., Lüdemann, H.-D., & Jaenicke, R. (1980b) *Biochemistry 19*, 1121-1126.
Schmid, F. X. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 387-400, Elsevier/North-Holland, Amsterdam.
Schmid, F. X. (1981) *Eur. J. Biochem.* (in press).
Schmid, F. X., & Baldwin, R. L. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4764-4768.
Teipel, J. W., & Koshland, D. E., Jr. (1971) *Biochemistry 10*, 792-805.
Vallee, R. B., & Williams, R. C. (1975) *Biochemistry 14*, 2574-2580.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1979) *Biochemistry 18*, 5567-5571.

Sulfhydryl and Histidinyl Residues in the Flavoenzyme Alcohol Oxidase from *Candida boidinii*†

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ABSTRACT: The flavoenzyme alcohol oxidase from *Candida boidinii* has about eight sulfhydryl residues per 75 000 molecular weight subunit. The enzyme is inactivated by treatment with *p*-(chloromercuri)benzoate at pH 7.5, but full enzymatic activity can be restored by treatment with excess β-mercaptoacetic acid. Complete inactivation of the enzyme requires reaction of all eight sulfhydryl residues per subunit. For any particular subunit, however, inactivation is an all-or-nothing event; i.e., slow reaction of a first sulfhydryl residue with *p*-(chloromercuri)benzoate is immediately followed by reaction of all remaining sulfhydryl residues of that subunit. The rate of inactivation shows saturation kinetics with respect to *p*-(chloromercuri)benzoate concentration, and substrates and products slow the rate of inactivation. The enzyme is also inhibited by treatment with Hg^{2+}, Cu^{2+}, and Ag^{+}, but no inactivation was found with *N*-ethylmaleimide, acrylonitrile, 5,5′-dithiobis(2-nitrobenzoate), or methyl methanesulfonate. Apparent time-dependent inactivation of alcohol oxidase with iodoacetate was found to be reversible on removal of excess inhibitor. Proposals for the function of sulfhydryl groups in alcohol oxidase are discussed. Alcohol oxidase is also rapidly inactivated by diethyl pyrocarbonate. The pH dependence of inactivation and absorbance changes on modification suggest that histidine residues are modified. Total inactivation of the enzyme occurs on reaction of four histidine residues per FAD coenzyme, but only one of these residues appears to be catalytically essential. [1-^{14}C]Diethyl pyrocarbonate was used to demonstrate that only histidine residues were modified. In the presence of either of the competitive inhibitors acetaldehyde or acetate, diethyl pyrocarbonate reacts with only 3 equiv of histidine in a fashion which suggests that the catalytically essential histidine is at the active site of the enzyme. Modification of alcohol oxidase with diethyl pyrocarbonate slightly alters the visible absorbance spectrum of the flavin coenzyme and prevents reduction by substrate methanol, consistent with a role for histidine at an early stage in catalysis.

There has been increasing interest in the use of microorganisms to produce protein for supplementing animal feed because the microorganisms can be rapidly produced by aerobic fermentation from raw materials which cannot be used directly by mammals (Hamer & Hamden, 1979). Methanol now appears to be promising as a growth substrate, and several bacteria and yeasts which can grow on methanol as sole carbon and energy source have been isolated (Sahm, 1977; Cardemil, 1978). Under such growth conditions, several yeasts of the *Candida* species have been found to produce a soluble alcohol

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oxidase which converts small primary alcohols to aldehydes without the use of pyridine nucleotide coenzymes (Fujii & Tonomura, 1972; Sahm & Wagner, 1973).

The alcohol oxidase (EC 1.1.3.13) from *Candida boidinii* contains FAD as the only cofactor and has a molecular weight of 600000 constructed from eight apparently identical subunits (Sahm & Wagner, 1973). In addition to small primary alcohols, the enzyme will also oxidize formaldehyde to formic acid (Sahm, 1975), reducing oxygen to hydrogen peroxide in all cases. Like the other enzymes which can oxidize ethanol, the *pro-1R* hydrogen is preferentially removed during reaction (Cardemil, 1975, 1978; Kraus & Simon, 1975). The enzyme is reported to be inhibited by organomercurials (Sahm & Wagner, 1973; Volfova, 1975; Dorgai, 1976), but little is known about the properties or the mechanism of the flavin-dependent alcohol oxidases. Based on model system studies, Bruice (1976) has proposed that the oxidation of alcohols by flavoenzymes should occur through radical intermediates rather than through carbanion intermediates, which have generally been accepted for the similar oxidation of α-hydroxy acids to α-keto acids (Bright & Porter, 1975; Walsh, 1978). This work continues the study of the mechanism of reaction of alcohol oxidase (Nichols & Cromartie, 1980) and reports that the enzyme contains unreactive thiol groups and a catalytically essential histidine residue at the active site of the enzyme.

Materials and Methods

N-Butylmaleimide was obtained from U.S. Biochemicals. Methanol, acetaldehyde, and acrylonitrile were purchased from Aldrich. Diethyl pyrocarbonate from Aldrich was distilled before use, and [1-^{14}C]diethyl pyrocarbonate was prepared by the procedure of Melchior & Fahrney (1970) from 0.25 mCi of [1-^{14}C]ethanol (California Bionuclear Corp., 5.86 Ci/mol). The distilled diethyl pyrocarbonate [bp 68–70 °C (3.5 mmHg)] had a specific activity of 2.2 μCi/mmol and was stored at −4 °C in anhydrous acetonitrile. Methyl methanesulfonate was prepared as described by Smith et al. (1975). Scintillation cocktail (TT-21) was purchased from Yorktown Research. All other biochemicals were obtained from Sigma.

Enzyme Purification and Assay. For some experiments, alcohol oxidase was purchased from Boehringer Mannheim and further purified by Sephadex G-200 chromatography on a 60 × 1.2 cm column equilibrated with 50 mM sodium phosphate at pH 7.5. Fractions of the highest specific activity were combined and concentrated with Millipore immersible molecular separators. Gel electrophoresis showed one major band with a slower moving impurity corresponding to less than 2% of the stain density of the major band. The enzyme was stored under N_2 at 4 °C in the absence of light, where it was stable for at least 3 weeks. For later experiments, the enzyme was purified from *C. boidinii* by published procedures (Sahm & Wagner, 1973; Tani et al., 1972). Gel electrophoresis gave essentially the same results as those for the commercial enzyme. No differences in the enzyme from either source were noted for experiments reported in this work.

The activity of the enzyme was measured with a model 5331 oxygen electrode from Yellow Springs Instrument Co. fitted in a 1-mL-volume reaction chamber. Assays were made in 50 mM sodium phosphate, pH 7.5, containing 50 mM methanol at 23 °C. Protein concentration was determined by the method of Lowry et al. (1951) or from the absorbance at 280 nm times 0.6, which gave the same value in milligrams per milliliter. Subunit concentrations were determined by using a molecular weight of 75000 per subunit. All kinetic studies were done with air-saturated buffers, and all kinetic constants are apparent values obtained with air-equilibrated solutions.

Reaction with Thiol Reagents. An aliquot of enzyme of known specific activity in 50 mM sodium phosphate buffer at pH 7.5 was mixed with the putative inactivator to give the desired concentration, and the solution was incubated in the dark at 23 °C. At intervals, 10-μL samples were withdrawn and diluted into 0.99 mL of the assay buffer containing 50 mM methanol. The initial rate of utilization of oxygen was measured immediately at 23 °C with the oxygen electrode system.

Reaction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with thiol groups on the enzyme was followed at 412 nm on a Beckman 25 K spectrophotometer at 30 °C. With the native enzyme, the reaction was followed for 8 h. Reaction of DTNB with denatured enzyme was followed for 30 min (when absorbance readings became constant) on enzyme samples which had been incubated at 30 °C in 2 M guanidinium chloride or 4 M urea for at least 30 min. Complete loss of enzymatic activity and shift of the wavelength maximum of the flavin coenzyme from 460 to 450 nm, characteristic of free FAD, indicated that denaturation was complete under these conditions. An extinction coefficient of 13600 M^{-1} cm^{-1} was used to convert the absorbance change to moles of thiol reacted. Inhibition of alcohol oxidase by iodoacetate was found to be completely reversible on removal of iodoacetate with a 40 × 1 cm column of Sephadex G-25 equilibrated with 50 mM sodium phosphate at pH 7.5.

The inactivation of alcohol oxidase with metals and organomercurials was studied in a similar fashion. It was first established that these compounds did not interfere with the O_2 electrode assay at the concentrations used in this study. The reactivation by thiols of alcohol oxidase inhibited with *p*-(chloromercuri)benzoate was done with β-mercaptoacetic acid at pH 7.5, since uncharged thiols such as β-mercaptoethanol or dithiothreitol could pass the semipermeable membrane of the oxygen electrode and interfered with the assay. Because β-mercaptoacetic acid was itself found to be a reversible inhibitor of the enzyme, control samples without organomercurial were also treated with an equivalent amount of β-mercaptoacetic acid before activity assays and comparison to enzyme samples inhibited with organomercurial. In studies of the titration of alcohol oxidase with *p*-(chloromercuri)-benzoate followed spectrophotometrically at 250 nm, 30 min of incubation at 30 °C was required before constant absorbance readings were obtained. A value of 7600 M^{-1} cm^{-1} was assumed for the extinction coefficient for formation of the Hg–S bond, and the concentration of *p*-(chloromercuri)-benzoate solutions was determined from the absorbance at 232 nm by using an extinction coefficient of 16900 M^{-1} cm^{-1} (Boyer, 1954).

Reaction with Diethyl Pyrocarbonate. To 83 μg of alcohol oxidase in 50 mM sodium phosphate at pH 7.5 and 0 °C was added an acetonitrile solution of diethyl pyrocarbonate. The acetonitrile concentration did not exceed 2% by volume, and the enzyme was found to be stable at 0 °C in this concentration of acetonitrile for up to 2 h. Higher concentrations of acetonitrile caused loss of activity, however. At appropriate times, the reaction was diluted into the assay buffer at 23 °C. For one series of experiments in which the modification was carried out at 30 °C, aliquots were quenched into an assay buffer of 200 mM imidazole at pH 7.5 to destroy residual diethyl pyrocarbonate. The concentration of inactivator in acetonitrile was determined from the absorbance at 242 nm after reaction with *N*-acetylhistidine using a molar extinction coefficient of 3900 M^{-1} cm^{-1} (Choong et al., 1977).

The pH-dependence studies of inactivation were conducted similarly with the pH values adjusted from 6.5 to 8.5. The pH did not change during the course of the experiment, and the enzyme lost negligible activity on standing in the absence of inactivator under these conditions. At lower pH, however, enzyme activity was rapidly lost, even at 0 °C.

Alcohol oxidase which had been inactivated to less than 5% residual activity was dialyzed against several changes of pH 7.5 phosphate buffer or was passed through a Sephadex G-25 column at 23 °C without recovering any activity. Reactivation was also not accomplished by addition of 10–100 mM hydroxylamine for periods of 15–45 min followed by Sephadex chromatography and specific activity determination. Equilibration of the Sephadex G-25 with substrate methanol also failed to lead to the recovery of activity from the inactivated enzyme.

Spectroscopic and Radioactive Experiments. For determination of the absorbance spectrum of the enzyme inactivated with diethyl pyrocarbonate, a 0.6-mL sample of enzyme which contained 5.7 nmol of subunits (subunit molecular weight 75000) was treated with 1.3 mM diethyl pyrocarbonate (137-fold molar excess over enzyme subunits) for 30 min and the absorbance spectrum taken against a blank containing the same amount of native enzyme and acetonitrile. Kinetic experiments on the rate of increase in absorbance at 242 nm due to ethoxyformylation of histidine were carried out at 30 °C. To an enzyme solution which was 0.46 mg/mL was added 1.3 mM diethyl pyrocarbonate, and the absorbance at 242 nm was followed for 5 min, after which time the absorbance remained constant. With the same conditions, the rate of loss of enzymatic activity was determined by the imidazole quench procedure. The experiment was repeated by using 0.77 mg/mL enzyme and 1.4 mM inactivator with the same correlation between the increase of absorbance at 242 nm and the loss of enzymatic activity being found. Experiments on the effect of acetaldehyde were done identically except that 10 mM acetaldehyde was present in both the absorbance increase and the loss of activity experiments.

A 0.1-mL sample of enzyme which contained 0.12 mg of alcohol oxidase was treated with 1 mM [1-^{14}C]diethyl pyrocarbonate for 10 min at 23 °C, when it retained 4% initial activity. The modification reaction was quenched with 10 mM imidazole, and the enzyme was passed through a Sephadex G-25 column. A sample of enzyme containing 0.27 nmol of active site by the 280-nm absorbance gave 1.0 nmol of diethyl pyrocarbonate incorporated. Corrected for the residual activity, this gave the number of labels per active site. The experiment was repeated in the presence of 10 mM acetaldehyde. Also, one inactivation was stopped at 48% residual activity but was treated identically otherwise. All scintillation counting was done on a Beckman LS-2150 instrument.

Results

Reaction with Thiol Reagents. Treatment of purified alcohol oxidase with 1 or 25 mM *N*-ethylmaleimide at 23 °C for up to 3 h caused no greater loss of activity than a control containing no inactivator. The same conclusion was reached for the more hydrophobic reagent *N*-butylmaleimide at 15 mM for 3 h. It was also found that 5.7 mM acrylonitrile or 10 mM iodoacetamide caused no time-dependent loss of activity over a 3-h period. Addition of 7.5 mM methyl methanesulfonate to alcohol oxidase caused a 7% increase in enzymatic activity, which was maintained for 3 h at room temperature. In view of the minor effects of the preceding reagents on enzymatic activity, no effort was made to determine whether reaction with the enzyme had occurred. Reaction with 1 or 2.7 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) also resulted in no loss of catalytic activity after 4 h at room temperature at pH 7.5. A sample of alcohol oxidase which was 1.6×10^{-5} M in subunits (by FAD content) developed no increase in absorbance at 412 nm on incubation with 1 mM reagent for 8 h, indicating that it had not reacted with sulfhydryl groups on the enzyme. Denaturation of the enzyme by treatment with 2 M guanidinium chloride or with 4 M urea for 30 min prior to addition of DTNB, however, did result in a rapid reaction with increase of the absorbance at 412 nm. From the absorbance increase and the enzyme subunit concentration, a value of 7.9 ± 0.1 sulfhydryl residues per subunit was found.

As previously reported by Dorgai (1976), iodoacetate causes substantial loss of enzymatic activity. Treatment of the enzyme with 10 mM iodoacetate at pH 7.5 and 23 °C caused a loss of 40% of the activity of a control after 1 h. Further kinetic studies of this reaction indicated that there was a rapid loss of activity on addition of iodoacetate to the enzyme that was complete within 15 min. Thereafter, the activity remained constant with time. Surprisingly for an alkylation expected to be irreversible, the *extent*, as well as the rate, of inactivation was dependent on the initial iodoacetate concentration: 40% inhibition with 10 mM reagent, 70% for 20 mM, and 80% for 30 mM. However, passage of a sample of alcohol oxidase inhibited with 30 mM iodoacetate to 20% residual activity through a column of Sephadex G-25 restored by enzyme to 103% of its initial activity. A similar observation was made by using dialysis to remove the inhibitor. It is clear that the inhibition of alcohol oxidase by iodoacetate is not due to covalent modification of a sulfhydryl, or other, residue on the enzyme. It was also found that the enzyme could be reduced in activity by sodium acetate, which was a competitive inhibitor with a K_I of 1.8 mM. In view of the inhibition by a simple carboxylic acid like acetate, it is suggested that the inhibition observed with iodoacetate may be due to simple, reversible binding to a site, possibly the active site, on the enzyme. However, iodoacetate does not function as a normal reversible inhibitor, since the inhibition takes approximately 15 min to develop. The reason for the time dependence of inhibition by iodoacetate is not clear and is under investigation.

Reaction with Metals. In contrast to the sulfhydryl reagents discussed above, alcohol oxidase was substantially inactivated by several metals and organomercury reagents. Treatment of alcohol oxidase with 100 μM *p*-(chloromercuri)benzoate at pH 7.5 and 23 °C led to a first-order loss of activity with a half-life of 11 min (Figure 1). The dependence of the first-order rate constant on the organomercurial concentration over the range from 60 to 260 μM reagent was not linear but gave rise to a saturation curve from which a maximum rate of inactivation of 0.046 s^{-1} and an apparent K_I of 5.4 mM could be obtained. The use of higher *p*-(chloromercuri)benzoate concentrations was precluded by the rapidity of the inactivation and the time required for the enzymatic assay. Addition of 13 mM methanol to the inactivation mixture containing 100 μM inactivator decreased the rate of inactivation, increasing the half-time for inactivation to 53 min. Ethanol also protected the enzyme, but *tert*-butyl alcohol did not. Since alcohol oxidase is able to convert formaldehyde to formic acid at an appreciable rate (Sahm, 1975), protection of the enzyme from inactivation by formate would be expected if the *p*-(chloromercuri)benzoate reaction occurred at the active site. This protection was observed (Figure 1). If a sample of alcohol oxidase totally inactivated by treatment with 83 μM *p*-(chloromercuri)benzoate was treated with 25 mM EDTA, no activity was recovered after 1 h. The presence of 2 mM EDTA

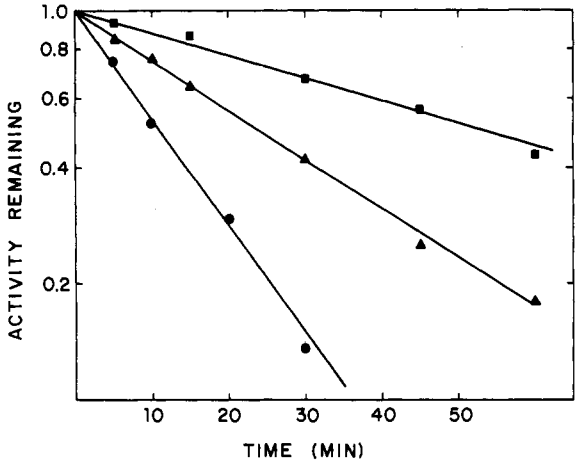

FIGURE 1: Rate of inactivation of alcohol oxidase with *p*-(chloromercuri)benzoate. Alcohol oxidase (0.4 mg/mL) was incubated with 100 μM inactivator at pH 7.5 and 23 °C in 50 mM sodium phosphate buffer (●). Aliquots were withdrawn at intervals and assayed after 100-fold dilution, and the relative rate was determined by comparison to an identical enzyme sample except without inhibitor. Effects of 13 mM methanol (■) or of 15 mM sodium formate (▲) on the rate of inactivation were also determined.

alone did not change the activity of the enzyme toward methanol as substrate. Likewise, Sephadex G-25 chromatography did not restore enzymatic activity. Treatment of the inactivated enzyme with 5 mM β-mercaptoacetic acid, however, led to complete recovery of activity as compared with a sample of enzyme not treated with inactivator but with β-mercaptoacetic acid. This experiment strongly suggests the inactivation arises through the formation of an organomercurial adduct with one or more sulfhydryl groups. In fact, adduct formation could be demonstrated directly by the increase in the 250-nm absorbance on inactivation of alcohol oxidase by *p*-(chloromercuri)benzoate. Reaction of 1.0 mL of a solution that was 13.4 μM in enzyme subunits with 0.1 mM *p*-(chloromercuri)benzoate for 75 min resulted in an increase of 0.78 in the 250-nm absorbance. With the assumption of a stoichiometric reaction between the organomercurial and protein thiol groups on the enzyme, 7.7 sulfhydryl groups per subunit can be calculated to have reacted. If the experiment were repeated except that the absorbance at 250 nm and the enzymatic activity were determined at intervals, a linear correlation between thiol groups modified by *p*-(chloromercuri)benzoate and enzymatic activity remaining was found (Figure 2). From this experiment, complete inactivation of alcohol oxidase by the organomercurial requires the modification of 7.8 equiv of sulfhydryl per enzyme subunit. For further exploration of the nature of the inactivation, a 4.0-mL sample of enzyme which was 74 μM in enzyme subunits was divided into two equal parts, one of which was treated with 45 μM *p*-(chloromercuri)benzoate for 2 h at 30 °C. At this point, the treated enzyme sample retained 35% of the activity of the untreated sample. The samples were separately made anaerobic with N_2, and the absorbance at 460 nm due to the FAD cofactor was determined. Excess methanol was added, and the solutions were incubated for 2 h at 30 °C. The decrease in the 460-nm absorbance due to reduction of the FAD cofactor of the *p*-(chloromercuri)benzoate-treated enzyme was only 40% of the absorbance decrease of the native, untreated enzyme on reduction by substrate. In a separate experiment, a similar 1:1 correspondence between the loss of enzymatic activity and the loss of the capacity of the FAD cofactor to be reduced by substrate was found with enzyme inhibited to 75% residual activity. As previously discussed by

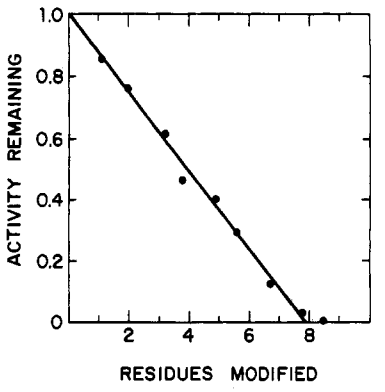

FIGURE 2: Correlation between the number of sulfhydryl residues modified by *p*-(chloromercuri)benzoate and the fractional activity remaining. To 1.0 mL of solution containing 14.2 μM enzyme subunits in 50 mM sodium phosphate at pH 7.5 and 30 °C was added *p*-(chloromercuri)benzoate to give 0.1 mM. At intervals, the absorbance at 250 nm and the enzymatic activity were determined. The absorbance increase was converted to equivalents of sulfhydryl reacted by using a molar extinction coefficient of 7600 M^{-1} cm^{-1}.

Table I: Inhibition of Alcohol Oxidase with Metals[a]

metal	conditions	activity lost (%)	reactivation
Hg^{2+}	1 mM, 30 min	100	100% with 5 mM $HSCH_2CH_2OH$
Cu^{2+}	1 mM, 15 min	95[b]	100% with 10 mM EDTA
Ag^{+}	0.1 mM, 5 min	100	none found
Cd^{2+}	2 mM, 2 h	5	

[a] The indicated reagents were added to a solution of alcohol oxidase at 23 °C to give a final enzyme concentration of 1.1 mg/mL. See text for details. [b] Inhibition of the enzyme by Cu^{2+} has been reported by Sahm & Wagner (1973).

Hellerman et al. (1965) for the inhibition of D-amino acid oxidase with metals, these kinds of experiments show that for any individual enzyme subunit inactivation is an all-or-nothing event. Since only *complete* inhibition of enzymatic activity should completely prevent reduction of the FAD cofactor by substrate, partial inactivation of alcohol oxidase by *p*-(chloromercuri)benzoate gives a mixture of fully active and fully inhibited subunits rather than a solution of equivalent, and partially inactivated, subunits. In no instance was precipitation of the inhibited alcohol oxidase observed.

Certain other transition metals also result in loss of enzymatic activity (Table I). Detailed studies of these inactivations were not conducted, but the effect of mercury, silver, and copper is consistent with inactivation of the enzyme by binding to or oxidation of sulfhydryl group(s). The notable failure of cadmium to inhibit the enzyme possibly indicates the absence of accessible dithiols in the enzyme which are critical to reactivity (Gaber & Fluharty, 1972).

Reaction with Diethyl Pyrocarbonate. Addition of 1.7 mM diethyl pyrocarbonate to alcohol oxidase in 50 mM sodium phosphate buffer at pH 7 and 0 °C leads to rapid loss of activity in a first-order process for which the semilogarithmic plot was linear down to 10% residual activity (Figure 3). The inactivation was found to be first order in enzyme and in inactivator with a rate constant of 0.42 M^{-1} s^{-1} at pH 7 and 0 °C. Although the instability of alcohol oxidase below pH 6.5 (Sahm & Wagner, 1973) confined studies to more basic conditions, the pH dependence of inactivation from 6.5 to 7.5 could be correlated with

$$\frac{1}{k_{2(obsd)}} = \frac{1}{k_2} + \frac{[H^+]}{k_2 K_a} \quad (1)$$

where k_2 is the second-order rate constant for modification

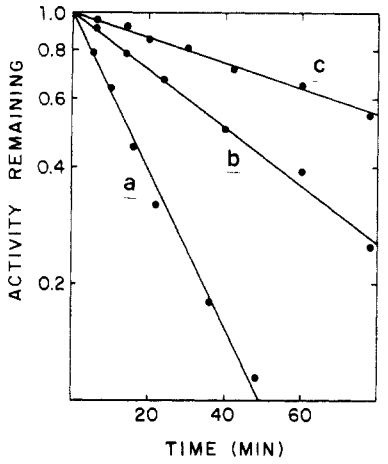

FIGURE 3: Rate of inactivation of alcohol oxidase with diethyl pyrocarbonate. The enzyme (0.6 mg/mL) in 50 mM sodium phosphate at pH 7.5 was treated with 1.7 mM reagent at 0 °C, and aliquots of the solution were assayed for residual enzymatic activity at intervals (a). The effect of the presence of 10 mM acetaldehyde (b) or of 25 mM sodium formate (c) on the rate of inactivation was also determined.

Table II: Inactivation of Alcohol Oxidase with [1-^{14}C]Diethyl Pyrocarbonate

expt	acetaldehyde concn (mM)	residual activity (%)	^{14}C labels incorporated
1	0	48	2.1
1	0	4	3.9
3	0	2	4.0
4	0	2	4.2
5	10	80	1.3
6	10	2	3.1
7[a]	0	0	0.3

[a] Experiment 3 after treatment with 75 mM hydroxylamine.

of an unprotonated residue with an apparent dissociation constant for the protonated form of K_a (Cousineau & Meighen, 1976). At 0 °C, k_2 was found to be 0.74 M^{-1} s^{-1}, and K_a was 1.1×10^{-7} ($pK_a = 7$). This pK_a is in the region expected for modification of histidine (Miles, 1977).

Extensive dialysis of the inactivated enzyme against 50 mM sodium phosphate, pH 7.5, at 4 °C or Sephadex chromatography with the same buffer at room temperature led to negligible (<5%) recoveries of activity. Similar treatment of enzyme partially inactivated to 45% residual activity returned enzyme with the same residual activity, demonstrating the failure to recover activity was not due to instability of the native enzyme to these conditions. It has been suggested that inactivation of an enzyme with diethyl pyrocarbonate probably occurs by modification of histidine residues if activity is recovered on treatment of the inactivated enzyme with hydroxylamine (Miles, 1977). Addition of 10–100 mM hydroxylamine to ^{14}C-labeled, inactivated alcohol oxidase, however, caused no recovery of activity, although most of the label was lost (Table II). Addition of hydroxylamine to partially inactivated enzyme led to rapid and complete loss of activity, which could not be restored on dialysis. Incubation of the native enzyme with 10 mM hydroxylamine caused a loss of 80% activity at 0 °C, so further reactivation experiments with hydroxylamine were not explored.

In the absence of evidence for histidine modification by hydroxylamine reactivation, spectroscopic experiments were undertaken. The *N*-carbethoxyhistidine produced by reaction of diethyl pyrocarbonate with histidine has an absorbance maximum at 240 nm (Miles, 1977). The difference spectrum obtained on treatment of a solution of 0.7 mg/mL alcohol

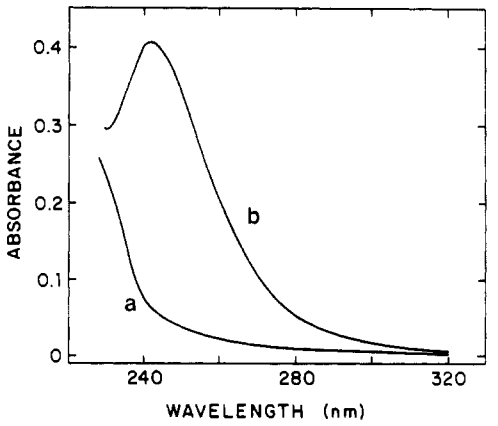

FIGURE 4: Ultraviolet difference spectrum for the inactivation of alcohol oxidase with diethyl pyrocarbonate. A 1.2-mL sample of alcohol oxidase (9.7 mg/mL) was divided equally between the sample and reference cuvettes in the spectrophotometer and the base line (a) recorded. To the sample cuvette was added 60 μL of a 13 mM diethyl pyrocarbonate solution in acetonitrile, and to the reference was added an equivalent volume of acetonitrile. After 30 min at 30 °C, the spectrum (b) was recorded. At this point, the enzyme solution in the sample cuvette retained less than 3% of the activity of the enzymatic solution in the reference cuvette.

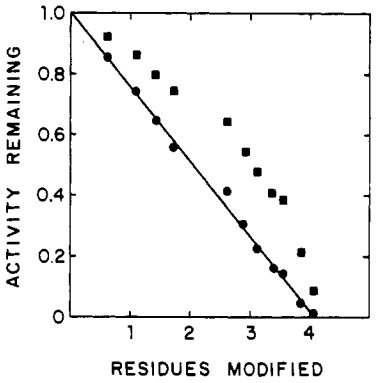

FIGURE 5: Correlation between the number of histidinyl residues of alcohol oxidase modified by diethyl pyrocarbonate and the fractional activity remaining. The sample cuvette contained the same solution as described for Figure 4. At intervals, the absorbance at 240 nm and the remaining enzymatic activity were determined. The change in absorbance was converted into histidines modified (see text). The data are presented in the form of a Tsou plot (see Discussion) for $i = 1$ (●) and for $i = 2$ (■).

oxidase with 1.3 mM inactivator is shown in Figure 4. This spectrum gives no indication of a reaction with tyrosine, for there is no decrease in the absorbance at 280 nm. The rate of increase in the 240-nm absorbance at pH 7.5 and 23 °C on the addition of 1 mM diethyl pyrocarbonate was determined for a solution containing 7.6 μM enzyme subunits. From these data, a value of 3900 M^{-1} cm^{-1} for the molar extinction coefficient of *N*-carbethoxyhistidine, and a molecular weight of 75 000 per subunit, the equivalents of histidine modified as a function of time could be determined. When the rate of loss of activity of the enzyme was measured under identical conditions, the correlation between loss of enzymatic activity and equivalents of histidine reacted shown in Figure 5 could be obtained. Extrapolation of the least-squares lines to zero enzymatic activity indicates that 4.1 histidine residues per subunit are modified before complete inactivation is achieved. However, application of the approach of Tsou (1962) indicates that only one of these is actually "essential" for catalysis. This analysis gives

$$a^{1/i} = 1 + \frac{s}{p} - \frac{m}{p} \qquad (2)$$

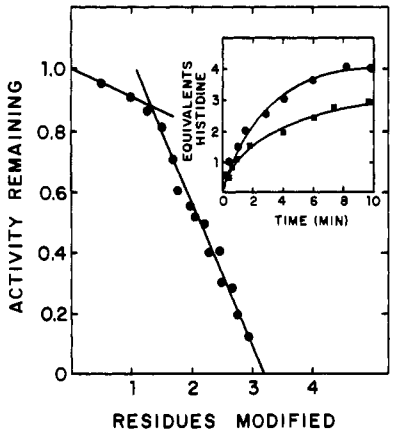

FIGURE 6: Correlation between the number of histidinyl residues modified in alcohol oxidase by diethyl pyrocarbonate in the presence of 10 mM acetaldehyde and the fractional activity remaining. Except for the presence of acetaldehyde, the experiment was conducted as described for Figure 5. Inset: Rate of ethoxyformylation of alcohol oxidase under the noted conditions in the absence (●) or in the presence (■) of 10 mM acetaldehyde.

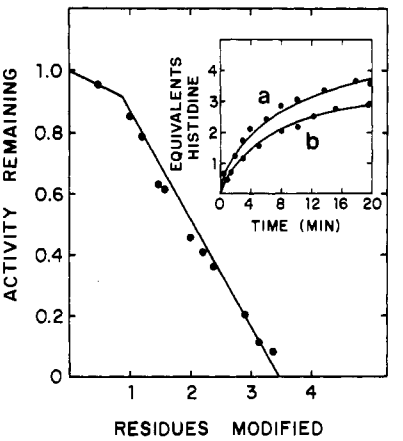

FIGURE 7: Correlation between the number of histidinyl residues modified in alcohol oxidase by diethyl pyrocarbonate in the presence of 70 mM acetate and the fractional activity remaining. Except for the acetate, the experiment was conducted as described in Figure 5. Inset: Rate of ethoxyformylation of alcohol oxidase under the noted conditions in the absence (a) or in the presence (b) of 70 mM acetate.

where a is the fraction of residual activity when m residues have been modified, s is the number of rapidly reacting but nonessential residues (zero for this case), and p is the number of moderately rapidly reacting groups (4 in this case) of which i are essential (Paterson & Knowles, 1972). A plot of $a^{1/i}$ vs. m will be linear only when i is assigned the integer corresponding to the number of essential residues per subunit. From Figure 5, it is clear that this holds for alcohol oxidase when $i = 1$ but not for $i = 2$. Larger values of i (3, 4) also do not give straight lines.

Although diethyl pyrocarbonate reacts preferentially with histidine residues on most proteins, reaction with other amino acids has occasionally been observed. Complete inactivation of the enzyme with radioactive inactivator followed by Sephadex gel filtration and determination of the equivalents of radioactivity incorporated showed that 4 equiv of reagent was incorporated per subunit (Table II). Since 4 equiv of diethyl pyrocarbonate is required to account for the reaction with histidine, no reaction with any other residue which gives a stable adduct is possible. Furthermore, partial inactivation of the enzyme with [1-^{14}C]diethyl pyrocarbonate leads to incorporation of radioactivity exactly to the extent predicted by the correlation of Figure 5 (Table II).

Inactivation of the enzyme in the presence of acetaldehyde causes a decrease in the rate of inactivation, as seen in Figure 3. Formate, which is a product of the alcohol oxidase catalyzed oxidation of formaldehyde (Sahm, 1975), also protects the enzyme from inactivation, as does the substrate methanol. When the effect of acetaldehyde on the correlation between fractional activity remaining and the modification of histidine residues was determined, the rather striking results shown in Figure 6 were obtained. The rate of increase of the 240-nm absorbance in the presence and absence of acetaldehyde, on which Figure 5 is based, is shown in the inset of Figure 6. Very similar results were obtained when the inactivation was carried out in the presence of the competitive inhibitor acetate (Figure 7). In both cases, modification of the most reactive histidine leads to very little loss of enzymatic activity, and one histidine appears to be almost completely protected from diethyl pyrocarbonate over the time span for total inactivation in the absence of competitive inhibitors. The remaining two histidines are modified, but more slowly in the presence of acetaldehyde or acetate than in their absence. Only one of these two histidine residues is essential [by the analysis of Tsou (1962) described earlier]. For the case of acetaldehyde, these results were confirmed by using radioactive diethyl pyrocarbonate, as noted in Table II.

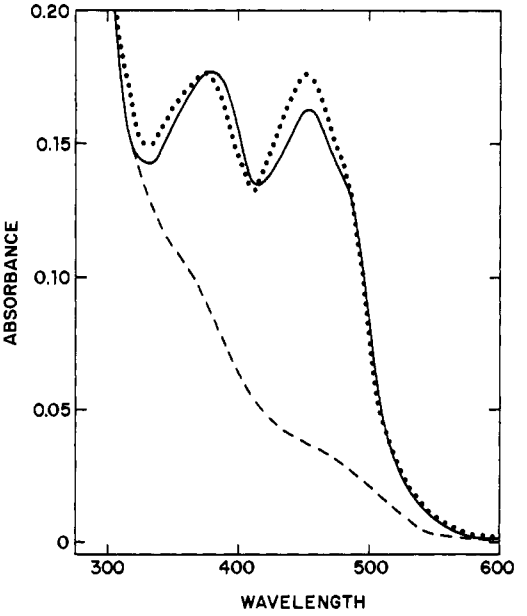

FIGURE 8: Visible absorbance spectrum of alcohol oxidase. The solid line represents the spectrum of native enzyme in 50 mM sodium phosphate buffer at 30 °C at a concentration of 1.95 mg/mL. After removal of O_2 by flushing with N_2, 1 μL of methanol was added, and the anaerobic enzyme solution was allowed to stand for 5 min before the spectrum was recorded (dashed line). An equivalent sample of native enzyme was inactivated by treatment with 1 mM diethyl pyrocarbonate at 30 °C for 15 min to give the fully inhibited enzyme with the spectrum shown by the dotted line.

On reaction with diethyl pyrocarbonate, the absorbance spectrum due to the FAD coenzyme changes slightly (Figure 8). Addition of methanol to the inactivated enzyme under anaerobic conditions did not cause reduction of the flavin chromophore even on extended incubation, in contrast to the instantaneous reduction of the native enzyme by methanol. Addition of sodium dithionite caused rapid bleaching of the flavin absorbance of both native and inactivated enzyme.

Discussion

Previous preliminary reports have suggested that alcohol oxidase from *Candida boidinii* can be inhibited by organomercurials, presumably by reaction with protein thiol groups

(Sahm & Wagner, 1973; Volfova, 1975; Dorgai, 1976). In this work, alcohol oxidase denatured with urea or with guanidinium chloride was found to have eight sulfhydryl residues per subunit of molecular weight 75 000. However, alcohol oxidase was not inactivated by a variety of electrophilic reagents commonly employed in protein chemistry as sulfhydryl-specific reagents. These reagents included acrylonitrile, *N*-methylmaleimide, *N*-butylmaleimide, and methyl methanesulfonate. Iodoacetate was found to inhibit the enzyme at high concentration (10–30 mM) by a time-dependent process, but the inhibition proved to be reversible on removal of iodoacetate by dialysis or by Sephadex chromatography. The origin of the time dependence of this inhibition has not yet been determined, but it is clear that iodoacetate does not inhibit by alkylation of a sulfhydryl, or other, residue on the enzyme. Surprisingly, acetate was found to be a potent competitive inhibitor of the enzyme (K_I = 1.8 mM). Aldehydes such as formaldehyde and trifluoroacetaldehyde, which exist primarily as hydrates in aqueous solution, are oxidized to carboxylic acids by alcohol oxidase, but the affinity of the enzyme for acetate is unexpectedly high. The known inhibition by formic acid of the growth of *C. boidinii* on methanol (Pilat & Prokop, 1976) may be due in part to inhibition of alcohol oxidase by formate, an inhibition which may have a regulatory function (Sahm, 1977).

Although alcohol oxidase has no nucleophilic sulfhydryl groups, treatment of the enzyme with Hg^{2+}, Cu^{2+}, or Ag^{+} caused a loss of enzymatic activity. The inhibition could be relieved by addition of thiols to the Hg^{2+}-treated enzyme or by the addition of EDTA to the Cu^{2+}-treated enzyme, suggesting reaction with a protein thiol group (Jocelyn, 1972). Enzymatic activity was also completely lost when the enzyme was treated with excess *p*-(chloromercuri)benzoate at pH 7.5. The inhibition could be reversed by thiol compounds but not by EDTA, behavior which indicates inhibition by formation of a covalent adduct of the organomercurial with sulfhydryl groups on the enzyme (Leavis & Lehrer, 1974). On the basis of the observed increase in the 250-nm absorbance due to formation of the Hg–S bond (Boyer, 1954), complete inactivation required reaction of *p*-(chloromercuri)benzoate with all eight sulfhydryl residues in each subunit. In fact, there was a linear correlation between the loss of enzymatic activity and the number of sulfhydryl residues modified until complete inhibition was achieved at eight residues modified. For each individual subunit, inactivation was found to be an all-or-nothing event; i.e., partially inactivated enzyme contained a mixture of fully active and fully inactive subunits with no partially active subunits present (Hellerman et al., 1965). A reasonable explanation for such behavior is that slow reaction of a first equivalent of an organomercurial with a sulfhydryl group with restricted accessibility leads to a conformational change in the protein which exposes the other seven sulfhydryl residues to rapid reaction with *p*-(chloromercuri)benzoate. Kinetic study of the inactivation showed saturation behavior with respect to increasing concentrations of the organomercurial group (apparent K_I = 5.4 mM). The usual interpretation of such a result is that *p*-(chloromercuri)benzoate must bind rapidly and reversibly to a site on the enzyme prior to a slower, and irreversible, reaction which leads to inactivation. Because the presence of alcohol substrates or formate, which bind at the active site, causes a substantial decrease in the rate of inactivation, the active site of the enzyme may be the site at which *p*-(chloromercuri)benzoate binds prior to inactivation. Perhaps the carboxyl group of the inhibitor directs the binding at the active site, so that *p*-(chloromercuri)benzoate functions as an active site directed affinity label.

The results on the inactivation of alcohol oxidase with *p*-(chloromercuri)benzoate can also be explained on the basis of the presence of different forms of the enzyme in solution. An all-or-none pattern of inactivation by excess *p*-(chloromercuri)benzoate would be expected if (i) the different forms of the enzyme have quite different reactivity toward the organomercurial, (ii) the equilibrium favored the less reactive species, and (iii) the interconversion of the forms were slow. The observed saturation kinetic behavior of the inactivation by the organomercurial would then arise naturally from the slow interconversion of the forms of the enzyme and would not require formation of a Michaelis complex prior to inactivation. The protective effects of substrates and of formate could arise from stabilization of the less reactive [toward *p*-(chloromercuri)benzoate] forms rather than from protection of an active site residue. Studies to distinguish between these two possible explanations and to determine whether subunit–subunit interactions are involved are being conducted. Whether any of the sulfhydryl groups of alcohol oxidase have catalytic functions cannot yet be determined, but sulfhydryl residues clearly have some role in maintaining the enzyme in a catalytically active state.

Diethyl pyrocarbonate inactivates alcohol oxidase rapidly at pH 7 and 0 °C. The pH dependence of inactivation shows the inactivation depends on the basic form of a residue with an apparent pK_a of 7, consistent with a histidine as the target residue. Spectroscopic investigation of the inactivation indicated that four histidine residues are modified before complete loss of activity is achieved (Figure 5). Evaluation of the relationship between the activity remaining in the enzyme and the number of residues modified showed that only one of the four reactive histidines is actually "essential" for catalysis. With [1-^{14}C]diethyl pyrocarbonate, it was found that 4 equiv of diethyl pyrocarbonate binds to the enzyme on complete inactivation. Since four residues are accounted for by reaction with histidine, no other groups are labeled by diethyl pyrocarbonate.

In the presence of acetaldehyde, a product and competitive inhibitor of the enzyme, the rate of reaction of diethyl pyrocarbonate with the enzyme is markedly reduced. Acetate, formate, and methanol also slow the inactivation, suggesting that the inactivation occurs by modification of the "essential" histidine residue in the active site of the enzyme. In the presence of acetate or acetaldehyde, the correlation between residual activity and equivalents of histidine modified changes dramatically from the same correlation in the absence of these inhibitors (Figures 6 and 7). In the absence of acetate or acetaldehyde, the four histidine residues which are labeled (one of which is essential) react at the same rate. In the presence of acetate or acetaldehyde, one of these residues is labeled at about the same rate as in their absence, with the loss of essentially no activity. Two other residues per subunit are modified more slowly, concomitant with the total loss of enzymatic activity. Thus, the essential residue is partially protected by the presence of competitive inhibitors of the enzyme. A fourth residue modified in the absence of acetaldehyde and acetate is not reactive within the time frame of the experiment if either is present. This residue may be in or near the active site of the enzyme without being required for catalytic activity, since the single essential residue has already been modified. These results, which depend upon the spectroscopic estimation of histidinyl residues modified, were substantiated with [1-^{14}C]diethyl pyrocarbonate.

After total inactivation of alcohol oxidase with diethyl pyrocarbonate, the flavin absorbance spectrum (Figure 8) undergoes significant changes in the 450-nm region. The reason for the absorbance increase in this region is not clear, but it suggests that at least some of the modified histidines interact with the flavin system. Like the native enzyme, inactivated alcohol oxidase is instantaneously reduced by sodium dithionite. However, the inactivated enzyme is not reduced by methanol under anaerobic conditions whereas the native enzyme loses the characteristic 450-nm flavin absorbance by such treatment. These experiments suggest that modification of the enzyme with diethyl pyrocarbonate interferes with bonding of substrates or with the catalytic process before the flavin is reduced, although the FAD coenzyme is still readily accessible to external reductants like dithionite.

On the basis of the studies reported in this work, it has not been possible to assign definite catalytic roles to either the sulfhydryl or the histidinyl residues of alcohol oxidase. For both kinds of amino acids, covalent modification leads to loss of enzymatic activity. However, there is a definite possibility that structural reorganization or conformational changes are important for this enzyme. The sulfhydryl and histidinyl residues could be essential primarily for maintenance of the proper configuration of the enzyme rather than be essential in the catalytic mechanism. Nevertheless, both of these amino acids are important in the proper functioning of the enzyme, and their roles can be delineated with further work.

References

Boyer, P. D. (1954) *J. Am. Chem. Soc. 76*, 4331.
Bright, H. J., & Porter, D. J. T. (1975) *Enzymes, 3rd Ed. 12*, 421.
Bruice, T. C. (1976) *Prog. Bioorg. Chem. 4*, 1.
Cardemil, E. (1975) *Biochem. Biophys. Res. Commun. 67*, 1093.
Cardemil, E. (1978) *Comp. Biochem. Physiol. B 60B*, 1.
Choong, Y. S., Shepherd, M. G., & Sullivan, P. A. (1977) *Biochem. J. 165*, 385.
Cousineau, J., & Meighen, E. (1976) *Biochemistry 15*, 4992.
Dorgai, L. (1976) *Proc. Hung. Annu. Meet. Biochem. 16*, 77.
Fujii, T., & Tonomura, K. (1972) *Agric. Biol. Chem. 36*, 2297.
Gaber, B. P., & Fluharty, A. L. (1972) *Bioinorg. Chem. 2*, 135.
Hamer, G., & Hamden, I. Y. (1979) *Chem. Soc. Rev. 8*, 143.
Hellerman, L., Coffey, D. S., & Neims, A. H. (1965) *J. Biol. Chem. 240*, 290.
Jocelyn, P. C. (1972) *Biochemistry of the SH Group*, p 77, Academic Press, London.
Kraus, A., & Simon, H. (1975) *Hoppe-Seyler's Z. Physiol. Chem. 356*, 1477.
Leavis, P. C., & Lehrer, S. S. (1974) *Biochemistry 13*, 3042.
Lowry, O. H., Rosebrough, N. H., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Melchior, W. B., & Fahrney, D. (1970) *Biochemistry 9*, 251.
Miles, E. W. (1977) *Methods Enzymol. 47*, 431.
Nichols, C. S., & Cromartie, T. H. (1980) *Biochem. Biophys. Res. Commun. 97*, 216.
Paterson, A. K., & Knowles, J. R. (1972) *Eur. J. Biochem. 31*, 510.
Pilat, P., & Prokop, A. (1976) *J. Appl. Chem. Biotechnol. 26*, 445.
Sahm, H. (1975) *Arch. Microbiol. 105*, 179.
Sahm, H. (1977) *Adv. Biochem. Eng. 6*, 77.
Sahm, H., & Wagner, F. (1973) *Eur. J. Biochem. 36*, 250.
Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766.
Tani, Y., Miya, T., Nichikawa, H., & Ogata, K. (1972) *Agric. Biol. Chem. 36*, 68.
Tsou, C.-I. (1962) *Sci. Sin. (Engl. Ed.) 11*, 1535.
Volfova, L. (1975) *Folia Microbiol. (Prague) 20*, 307.
Walsh, C. T. (1978) *Annu. Rev. Biochem. 47*, 881.