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Inactivation of L-Lactate Monooxygenase with 2,3-Butanedione and Phenylglyoxal†

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ABSTRACT: L-Lactate monooxygenase from *Mycobacterium phlei* is inactivated by reaction either with 2,3-butanedione in borate or in 2,6-lutidine buffer or with phenylglyoxal in 2,6-lutidine buffer. The inactivation with 2,3-butanedione in borate buffer is irreversible in the presence of excess borate, but essentially complete recovery of activity occurs on exchange of phosphate for boate buffer. In 50 mM borate, inactivation with 2,3-butanedione exhibits saturation kinetics with respect to increasing concentrations of 2,3-butanedione, whereas second-order kinetics for inactivation are seen in 200 mM borate. In 2,6-lutidine buffer, the inactivation is rapid, irreversible on change of buffer, and second order overall. Complete inactivation of the enzyme by phenylglyoxal in 2,6-lutidine buffer occurs on incorporation of 2 equiv of phenylglyoxal per subunit, but only one arginyl residue per subunit is modified. The inactivation is irreversible and second order in phenylglyoxal. There is substantial protection from inactivation in the presence of D-lactate, a competitive inhibitor of the enzyme. It is suggested that an arginyl residue in the active site in L-lactate monooxygenase is involved in the binding of the carboxyl group of substrates to the enzyme. An explanation for the unusual kinetics of inactivation with 2,3-butanedione in borate and with phenylglyoxal in 2,6-lutidine is offered.

Flavin-dependent enzymes which catalyze the oxidation of alcohol or amine groups in biological molecules are especially important because they couple the two-electron oxidation chemistry of such substrates to the one-electron chemistry of molecular oxygen or of metalloproteins in the electron-transport chain (Walsh, 1980). There has been considerable mechanistic study of these enzymes, focused mainly on the early steps in catalysis in which the substrate is oxidized and the flavin coenzyme is reduced (Bright & Porter, 1975; Bruice, 1975; Walsh, 1978). At least for the oxidation of alcohol and amine substrates having an adjacent carboxyl group considerable evidence has been accumulated that a carbanion intermediate or transition state is generated by removal of a proton from the carbon being oxidized (Walsh et al., 1971; Porter et al., 1973; Bruice, 1975; Ghisla & Massey, 1977). Studies on the enzyme L-lactate monooxygenase have been important in the development of this proposal, and a considerable body of research on the kinetics, spectral and fluorescence properties, and mechanism of this enzyme is available. Although the enzyme is an internal monooxygenase which catalyzes the four-electron oxidation of L-lactate to acetate and carbon dioxide with the reduction of O_2 to water, the mechanism is well established to proceed by oxidation of lactate to pyruvate, which is subsequently oxidatively decarboxylated (Lockridge et al., 1972). Recently, evidence of a covalent adduct of the substrate glycollate and N(5) of the flavin mononucleotide (FMN) coenzyme as a catalytically competent intermediate has been reported (Massey et al., 1980; Ghisla & Massey, 1980).

In view of the extensive mechanistic study of L-lactate monooxygenase, it is surprising that so little information on active-site amino acid residues necessary for catalysis is available. Modification by diethyl pyrocarbonate was used by Choong et al. (1977) to show that lactate monoxygenase from *Mycobacterium smegmatis* has a single histidine residue in the active site essential for normal catalysis. The binding of substrates and inhibitors and the chemical reactivity of the coenzyme were not altered by reaction of the enzyme with diethyl pyrocarbonate, and the suggestion was made that the essential histidine residue may function as the base which generates the substrate carbanion during catalysis. In addition to the carboxylate group of substrates, lactate monooxygenase has been found to bind a wide variety of anions at its active site, indicating the presence of one or more cationic residues (Massey et al., 1969; Lockridge et al., 1972; Ghisla & Massey, 1977). Choong et al. (1978) have reported that lactate monooxygenase from *M. smegmatis* is inactivated by treatment with 2,4-dinitrofluorobenzene and that a histidine and a lysine residue in the active site are modified. Since this modified enzyme was unable to bind anions, they suggested the lysine residue might be involved in the binding of anions to the enzyme. However, about 50% of the FMN coenzyme in the modified enzyme was slowly reduced by lactate and formed a complex with bisulfite. The lactate monooxygenase from *Mycobacterium phlei* (Takemori & Katagiri, 1975) appears to be similar to the enzyme from *M. smegmatis* in most respects except subunit structure (six for the *M. phlei* and eight for the *M. smegmatis* enzyme), but it has been less well studied. In connection with a study of some unusual hydrolytic activities of the *M. phlei* enzyme, an investigation of the residue(s) involved in the binding of anions to the active site of this enzyme was begun.

Through the use of the arginine-selective reagents 2,3-butanedione and phenylglyoxal, arginine residues have been implicated in the binding of anions, especially derivatives of phosphate, to a variety of enzymes (Riordan et al., 1977; Schneider, 1978). The flavoenzymes D-aspartate oxidase and

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D-amino acid oxidase have been found to have essential arginine residues presumably involved in the binding of anions at the active site (Crifo et al., 1977; Nishino et al., 1980). Therefore, the function of arginine residues in lactate monooxygenase was studied with both 2,3-butanedione and phenylglyoxal. With both reagents, unusual kinetic behavior was observed, and a single arginine residue at the active site was found to be required for the binding of anionic substrates.

Experimental Procedures

Materials. L-Lactate monooxygenase from *Mycobacterium phlei* was isolated and purified as described by Takemori & Katagiri (1975). The crystalline enzyme was stored in 80% saturated ammonium sulfate at 4 °C in the dark. Before use, the enzyme was dialyzed against the appropriate buffer and its concentration determined either by the absorbance at 280 nm with $E_{280}^{1\%} = 20.4$ and a molecular weight of 250 000 (Takemori & Katagiri, 1975) or by the method of Lowry et al. (1951) with bovine serum albumin as standard. Tris, D-lactate, L-lactate, and bovine serum albumin were purchased from Sigma. Phenylglyoxal (recrystallized from water before use), 2,6-dimethylpyridine, and D_2O were obtained from Aldrich. 2,3-Butanedione from Eastman was vacuum distilled and stored under nitrogen. Inorganic chemicals were reagent grade. Scintillation cocktail (TT-21) was purchased from Yorktown Research.

[^{14}C]Phenylglyoxal was prepared from [^{14}C]acetophenone (California Bionuclear Corp., 0.2 Ci/mol) by the procedure of Riley & Gray (1943). Repeated recrystallization from water gave [^{14}C]phenylglyoxal (0.12 Ci/mol) which showed a single spot on silica gel thin-layer chromatography (TLC) with 2:1 benzene/methanol as solvent and a UV spectrum identical with that of purified, commercial phenylglyoxal.

Inactivation with 2,3-Butanedione. Initial studies indicated the inactivation of L-lactate monooxygenase with 2,3-butanedione was slow below pH 8, and a pH of 8.4 was adopted for subsequent studies. Because of the interaction of 2,3-butanedione with borate buffer which causes a significant pH change (Riordan, 1973), solutions of 2,3-butanedione in each buffer system studied were prepared, and the pH was adjusted to 8.4. The appropriate amount of such a stock solution was added to 0.7 mmol of L-lactate monooxygenase in the same buffer at pH 8.4 and 23 °C to give a final volume of 200 μL. The inactivation was monitored by withdrawing 10-μL samples at intervals and quenching into 2.0 mL of 50 mM sodium borate at pH 8.4 containing 19 mM L-lactate. The activity of the enzyme was determined from the rate of consumption of oxygen measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co.). Although L-lactate monooxygenase is most active near pH 6 (Takemori et al., 1968), it was assayed at the pH of the inactivation to avoid changes in the pH of the assay buffer when aliquots of differing buffer concentration were added. For experiments in which the effects of inhibitors on the rate of inactivation were measured, the enzyme was incubated for 5 min with the compound before the inactivation was begun. Reversibility of the inactivation was determined by Sephadex chromatography (35 × 1 cm column of G-25) of the enzyme in the buffer being examined. Native L-lactate monooxygenase was found to be completely stable to this procedure in all buffer systems studied.

Inactivation with Phenylglyoxal. The inactivations were conducted in 50 mM 2,6-lutidine buffer at pH 8.4 as described for the study with 2,3-butanedione. In the case of the study of the pH dependence of inactivation, 100 mM 2,6-lutidine at the desired pH was used. Usually the enzyme was assayed in 50 mM 2,6-lutidine buffer at pH 8.4 and 23 °C in the presence of 19 mM L-lactate, but the pH of the assay solution was lowered to 7.5 when the effect of the competitive inhibitor D-lactate was studied. This change was necessary to give reliably measurable activity values for the enzyme which was both inhibited by D-lactate and partially inactivated by phenylglyoxal. Reversibility of the inactivation by phenylglyoxal was studied by Sephadex chromatography or by dialysis with 50 mM sodium phosphate at pH 7.5. Incorporation of [^{14}C]phenylglyoxal into the enzyme was studied under conditions identical with those for the kinetic studies. The labeled, partially inactivated enzyme was separated from [^{14}C]-phenylglyoxal on a Sephadex column. The eluted solution was assayed for activity and protein concentration (Lowry procedure, which was not influenced by the modification), and the incorporation of radiolabel was measured with a Beckman LS-3150P liquid scintillation counter.

The modification of the basic amino acid residues by phenylglyoxal was examined by amino acid analysis. After inactivation with phenylglyoxal, the enzyme was separated from the inactivator by Sephadex chromatography in 10 mM borate buffer at pH 7.5. After analysis for protein concentration and residual activity, the sample was hydrolyzed with 6 N HCl at 110 °C for 24 h in evacuated, sealed ampules. Amino acid analysis was performed on the long column of a Glenco Model 100-AS amino acid analyzer so that only tyrosine, phenylalanine, lysine, histidine, and arginine were examined. Instrumental conditions were chosen to optimize the height and symmetry of the arginine peak, and color values for the four amino acids were obtained with standards just prior to studies on the hydrolyzed protein. Native L-lactate monooxygenase was also run for direct comparison; the phenylalanine peak was used to correlate the two samples with each other and with the reported amino acid analysis of Takemori et al. (1974).

Absorption spectra of native and phenylglyoxal-inactivated L-lactate monooxygenase were measured with a Beckman 25K spectrophotometer. The molecular weight of the inactivated enzyme was determined by comparison with native enzyme on a 55 × 1 cm column of Sephadex G-200 at 4 °C eluted with 0.05 M sodium phosphate buffer at pH 7.5.

Results

Inactivation with 2,3-Butanedione in Borate. The data of Figure 1A show that the relatively slow inactivation of L-lactate monooxygenase with various concentrations of 2,3-butanedione (15–300-fold molar excess over enzyme subunit concentration) in 50 mM sodium borate at pH 8.4 and 23 °C is pseudo first order in all cases. Complete inactivation of the enzyme could be obtained with longer incubation times. A plot of the first-order rate constants for inactivation against the concentration of butanedione employed (Figure 1B) is distinctly nonlinear. Such behavior is characteristic of saturation kinetics and is usually taken as evidence for the formation of a complex of the inactivator and the enzyme prior to the inactivation event. From a double-reciprocal plot of the data in Figure 1B, an apparent K_I of 23 mM and maximum velocity for inactivation of 0.038 min^{-1} could be calculated. However, the observed saturation behavior becomes pronounced as the butanedione/borate ratio approaches one, and borate buffer has generally been found to enhance the reactivity of butanedione toward proteins. Therefore, the observed saturation kinetics for inactivation could be an artifact due to an interaction between butanedione and borate.

Riordan (1973) has shown that borate buffer reacts with butanedione to give unidentified products and to lower the concentration of the inactivator available for reaction. Addition of borate to aqueous butanedione lowers the pH of the

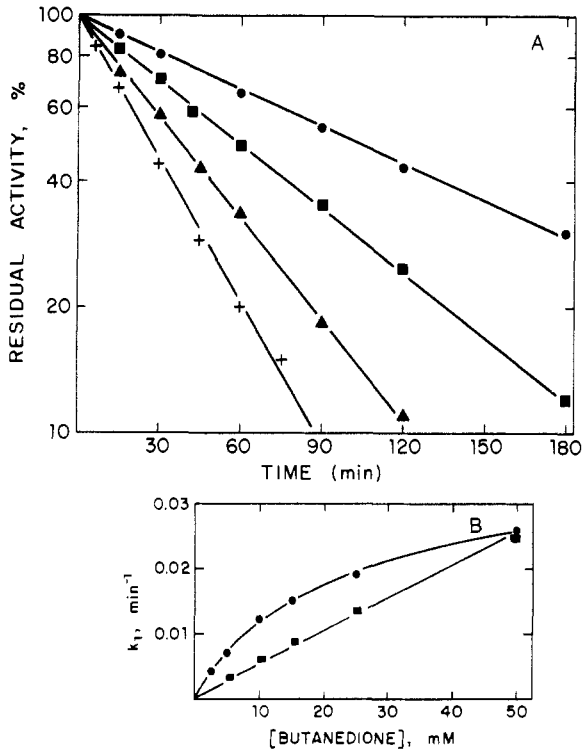


FIGURE 1: (A) Inactivation of L-lactate monooxygenase with 2,3-butanedione in 50 mM borate buffer at pH 8.4 and 23 °C. Reaction mixture contained 0.2 mg of enzyme and the indicated concentrations (mM) of 2,3-butanedione: (●) 5; (■) 10; (▲) 25; (+) 50. At intervals, aliquots were removed and assayed for activity against L-lactate with an oxygen electrode system. Initial activity was determined just after addition of the inactivator. (B) Relationship between the pseudo-first-order rate constant for inactivation from (A) and the concentration of 2,3-butanedione employed: (●) with 50 mM borate; (■) with 200 mM borate.

solution and decreases the 408-nm absorbance due to the butanedione. However, a quantitative measure of the butanedione/borate interaction is not available. To determine if the observed saturation kinetics for the inactivation of L-lactate monooxygenase could be due to such a reaction, the effect of borate on the proton NMR spectrum of butanedione was examined. In D_2O, butanedione shows resonances at δ 2.2 and 1.4 for methyl groups adjacent to a carbonyl group or to a *gem*-diol of the monohydrate, respectively (Greenzaid et al., 1967; Hooper, 1967). Addition of borate in D_2O at pH 7.5 (uncorrected apparent value) gives rise to new signals in the δ 1.0–1.5 region and decreases the signals for butanedione and its hydrate beyond the effect of dilution (Figure 2). If it is assumed that borate does not alter the equilibrium between the dione and *gem*-diol forms of 2,3-butanedione in solution and that the signals in the δ 1.0–1.5 region are due to the formation of borate complexes with butanedione, an approximate equilibrium constant between butanedione and borate and their complex can be determined, since no other resonances except for the HOD solvent were observed. The *relative* concentrations of free butanedione (both dione and hydrate forms) and of butanedione complexed with borate were determined by integration of the appropriate resonances. Since the peak for the hydrate at δ 1.4 was not clearly separated from the new signals due to the borate complex, the total integration for uncomplexed butanedione was taken as 1.56-fold the integration of the δ 2.2 (obtained from the ratio of δ 1.4 and 2.2 signals in the absence of borate). The integration for complexed butanedione was determined from the integration for the δ 1.0–1.5 region less the integration for the hydrate. From the known total concentrations of borate and butanedione present, an apparent association constant of 3.5 ± 0.1 M^{-1} (average for six butanedione and borate concentrations) for eq 1 at pH 7.5 and 23 °C can be calculated. The assumed

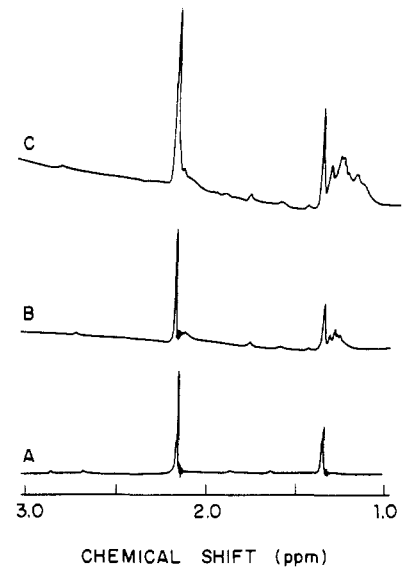


FIGURE 2: Nuclear magnetic resonance spectra of 0.6 mM 2,3-butanedione in D_2O at pH 7.5 (apparent) in the presence of borate buffer: (A) 0 mM; (B) 167 mM; (C) 250 mM. The spectra were analyzed as discussed in the text.

2,3-butanedione (dione and hydrate) + borate ⇄ butanedione–borate complexes (1)

1:1 stoichiometry of eq 1 under these conditions is supported by the invariance of the equilibrium constant over a range of 10–250 mM borate. No attempt has been made to determine the structure of the adduct between butanedione and borate which is responsible for the resonances at δ 1.0–1.5. In fact, the number and width of these lines may suggest that many complexes with similar structures can be formed. It is possible that butanedione/borate complexes which cannot be discerned by NMR are also formed. For this reason, and because of the assumptions made in its determination, the value of the equilibrium constant for eq 1 must be regarded as an approximation. However, it is consistent with the findings by Riordan (1973) that most of the butanedione in a 0.5 M solution of borate will be present as a complex. From the observed equilibrium constant, 63% of the butanedione should be complexed at 0.5 M borate, but only 17% should be complexed at 0.05 M borate. This small an effect could not be the entire cause for the nonlinear kinetic behavior seen in Figure 1B.

The inactivation experiments were repeated in the presence of 200 mM borate. As before, the inactivation is pseudo first order, but a plot of the first-order rate constant for inactivation against the concentration of butanedione is linear Figure 1B). If the saturation kinetic behavior seen for inactivation in 50 mM borate were due to loss of the inactivator by complexation with borate, an even more pronounced deviation from second-order kinetics for inactivation would be expected in 200 mM borate. An explanation for these unusual inactivation kinetics, based on the two-step nature of the reaction of butanedione with arginine residues in borate buffer (Riordan, 1973), is discussed below. From the inactivation data in 200 mM borate at pH 8.4 and 23 °C, a rate constant of 0.46 M^{-1} min^{-1} can be calculated. With 50 mM butanedione the inactivation in 200 mM borate is approximately as fast as in 50 mM borate. A plot of the log of the first-order rate constant for inactivation against the log of the butanedione concen-

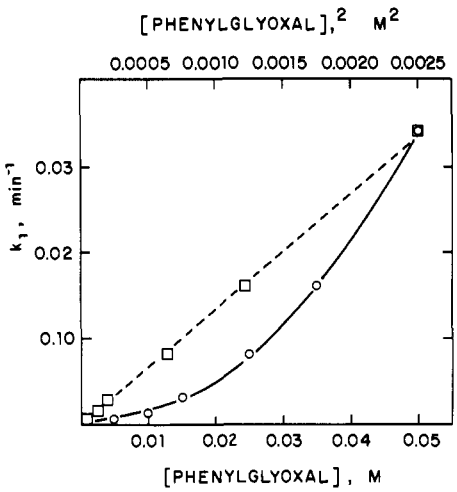


FIGURE 3: Relationship between the pseudo-first-order rate constant for inactivation of L-lactate monooxygenase by phenylglyoxal in 50 mM lutidine buffer at pH 8.4 and 23 °C and the concentration (○) or the square of the concentration (□) of phenylglyoxal employed.

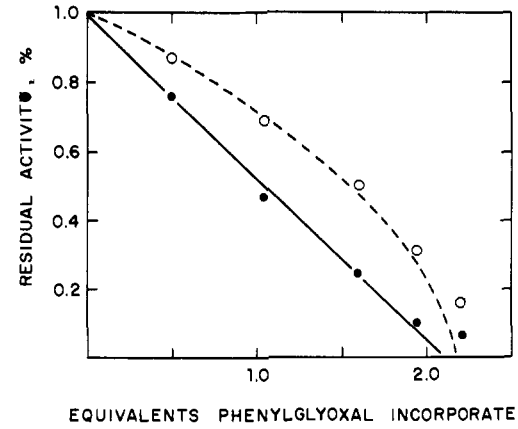


FIGURE 4: Correlation between enzyme activity remaining and equivalents of [^{14}C]phenylglyoxal incorporated into the protein. Inactivation was conducted with 4 mg of L-lactate monooxygenase and 14.5 mM [^{14}C]phenylglyoxal in 50 mM lutidine buffer, pH 8.4, 23 °C. (●) Activity to the first power; (○) activity to the one-half power. Procedure is discussed in the text.

tration in 200 mM borate has a slope of 0.80. Inactivation in the absence of light gave the same results. The inactivation of L-lactate monooxygenase with butanedione is irreversible so long as borate buffer is present. Enzyme with a specific activity of 2.65 μmol min^{-1} mg^{-1} was incubated with 50 mM butanedione in 50 mM borate until 5% of the initial activity remained. Sephadex chromatography in 50 mM borate gave eluted enzyme with a specific activity 4% of the native enzyme. On the other hand, Sephadex chromatography of a sample of the inactivated enzyme in 50 mM sodium phosphate buffer at pH 8.4 caused a recovery of 92% of the original activity. The kinetics of reactivation were not studied, but it is evident that borate is required to prevent reversal of the inactivation.

Inactivation with 2,3-Butanedione in Lutidine Buffer. Although most studies of the reaction of butanedione with proteins have been conducted in borate buffer, there are several reports in which other buffer systems have been used (Ehrlich & Coleman, 1977; Kremer et al., 1980). Preliminary experiments showed that inactivation of L-lactate monooxygenase did not occur in phosphate or in imidazole buffers. However, in 50 mM 2,6-dimethylpyridine (lutidine) buffer at pH 8.4, the enzyme was inactivated even at 0 °C. In this buffer, there is a linear relationship between the rate of inactivation and the concentration of butanedione from which a second-order rate constant of 0.31 M^{-1} min^{-1} at 0 °C can be determined. Changing the concentration of lutidine buffer had no significant effect on the rate of inactivation. A plot of the log of the first-order rate constants against the log of the butanedione concentration had a slope of 0.92. Sephadex chromatography of the inactivated enzyme in 50 mM lutidine or in 50 mM phosphate buffer at pH 8.4 or at pH 7.5 did not lead to the recovery of any significant activity.

Inactivation by Phenylglyoxal in Lutidine. Treatment of L-lactate monooxygenase with phenylglyoxal in 50 mM lutidine at 23 °C caused a time-dependent loss of activity. The inactivation became more rapid as the pH was increased from 7.9 to 9, and a pH of 8.4 was adopted for further study. The inactivation appears to be pseudo first order (20–300-fold molar excess of phenylglyoxal over enzyme subunits). A plot of the first-order rate constants for inactivation against the concentration of phenylglyoxal is markedly nonlinear (Figure 3). However, if the data are replotted by using the *square* of the phenylglyoxal concentration, a straight line is obtained (Figure 3), indicating the inactivation is first order in enzyme but second order in phenylglyoxal. Consistent with this kinetic order, a plot of log (rate of inactivation) vs. log [phenylglyoxal] had a slope of 1.9. Similar observations of the inactivation of L-lactate monooxygenase from *M. smegmatis* by 2,4-dinitrofluorobenzene have been reported by Choong et al. (1978).

The inactivation by phenylglyoxal cannot be reversed by removal of excess inactivator. A sample of enzyme treated with 20 mM phenylglyoxal until the residual activity was less than 1% the original value was chromatographed on Sephadex G-25 at 4 °C in 50 mM phosphate buffer at pH 8.4. The recovered enzyme still had a specific activity less than 1% that of native enzyme, and this activity did not increase on standing at 4 °C for 48 h. When the enzyme was allowed to react with 14.5 mM [^{14}C]phenylglyoxal (0.123 Ci/mol) in 50 mM lutidine buffer at pH 8.4, radioactivity was incorporated into the protein. At intervals, aliquots from the inactivation solution were chromatographed on Sephadex G-25, and the specific activity and incorporated of radioactivity were measured for the eluted enzyme. A plot of the specific activity remaining vs. the equivalents of [^{14}C]phenylglyoxal incorporated per mole of subunit is shown in Figure 4. Extrapolation of the line from data above 8% residual activity shows that 2.12 equiv of phenylglyoxal are required for total inactivation. However, as complete inactivation is approached, more extensive labeling of the enzyme is observed. Essentially all of the radioactivity incorporated into the enzyme was associated with the apoprotein and not with the FMN coenzyme. On the basis of the work of Takahaski (1968), it has generally been assumed that phenylglyoxal reacts with arginine residues to give an adduct containing two phenylglyoxal residues per arginine. If this were the case with L-lactate monooxygenase, the data in Figure 4 would indicate that only one arginine residue in the enzyme was modified. However, there are a number of instances in which a 1:1 ratio of phenylglyoxal to arginyl residues modified has been found (Borders & Riordan, 1975; Werber et al., 1975; Berghauser, 1977; Philips et al., 1978, 1979). Therefore, the effect of inactivation by phenylglyoxal on the basic amino acids were explored. The data of Table I show that one arginine is lost on inactivation of the enzyme but that the lysine content is not altered. Because of the difficulty in precisely quantitating small changes in amino acid content, special care was taken with the amino acid analyses. With standard solutions of arginine, it was found that the 4% difference between the loss of one or two of the 26 arginyl residues of the enzyme could be reliably determined. Since only one arginine is lost and two phenylglyoxal residues are incorporated into the en-

Table I: Amino Acid Analysis of Native and Phenylglyoxal-Inactivated L-Lactate Monooxygenase[a]

enzyme	activity (%)	Phe[b]	Lys	His	Tyr	Arg
native[c]	100	15.1	13.4	11.8	16.2	26.3
native	100	15.1	13.0	12.0	17.0	26.0
modified	7	15.1	13.0	12.2	16.8	25.1

[a] Inactivation with 20 mM phenylglyoxal as described in Figure 1A. The residues are calculated per subunit (56 000 g of protein). [b] Used to scale the native and modified enzyme analyses to each other and to the work of Takemori et al. (1974). [c] From Takemori et al. (1974).

zyme, the adduct formed in the inactivation of L-lactate monooxygenase has the 2:1 stoichiometry most often observed for this type of inactivation.

In the presence of competitive inhibitors of the enzyme, the rate of inactivation by phenylglyoxal is markedly reduced. D-Lactate is known to be a competitive inhibitor of the enzyme (Takemori et al., 1968). The presence of 20 mM D-lactate causes a 10-fold reduction in the rate of inactivation by 15 mM phenylglyoxal. From data not shown, it was found that anions such as phosphate, perchlorate, and sulfate, which are known to bind at the active site of L-lactate monooxygenase (Lockridge et al., 1972), also slow the inactivation by phenylglyoxal.

Inactivation of the enzyme with phenylglyoxal did not significantly change the visible absorption spectrum of the enzyme due to the FMN coenzyme, indicating that the coenzyme was not altered by the inactivation. If totally inactivated enzyme was treated with a 200-fold molar excess of substrate L-lactate under anaerobic conditions, no change in the visible absorption spectrum of the enzyme occurred over a 2-h period. On similar treatment of native L-lactate monooxygenase, the 450-nm absorbance due to the flavin is instantaneously bleached. However, addition of sodium dithionite to give 0.5 mM rapidly bleached both native and inactivated enzyme to the same featureless spectrum. The L-lactate monooxygenase from *M. smegmatis* has been found to be irreversibly inactivated by the suicide substrate 2-hydroxy-3-butynoic acid by a process which causes a significant alteration in the structure and in the absorption spectrum of the FMN coenzyme (Walsh et al., 1972; Ghisla et al., 1976). Native L-lactate monooxygenase from *M. phlei* undergoes essentially identical changes with the acetylenic hydroxy acid (T. Cromartie, unpublished experiments). In contrast, incubation of the phenylglyoxal-modified enzyme with hydroxybutynoate under aerobic or anaerobic conditions caused no alteration of the absorption spectrum of the enzyme. The inactivated enzyme also failed to bind inorganic phosphate at the active site, since the addition of 0.1 M phosphate did not induce the perturbations in the flavin absorption spectrum which occur with native enzyme from *M. smegmatis* (Lockridge et al., 1972 and from *M. phlei* (T. Cromartie, unpublished experiments). The uncorrected fluorescence emission of the inactivated enzyme (excitation 290 nm) had a maximum at 332 nm and was essentially identical with that for native enzyme. As judged by identical behavior on Sephadex G-200 chromatography, native and modified enzyme had the same molecular weight.

Discussion

The development of 1,2-dicarbonyl compounds such as butanedione, phenylglyoxal, and cyclohexanedione as arginine-selective reagents in protein modification studies has allowed the identification of arginine as a catalytically essential residue in a large number of enzymes (Riordan et al., 1977; Schneider, 1978). L-Lactate monooxygenase is inactivated by 2,3-butanedione in borate buffer. So long as borate was present, the inactivation was not reversed by removal of excess butanedione. However, essentially complete activity could be recovered by exchange of the borate buffer with phosphate in the time necessary to put the enzyme through a Sephadex G-25 column. In addition to arginine, butanedione conceivably could react with cysteine, histidine, and lysine residues in the enzyme to cause loss of activity. The exchange of phosphate for borate buffer would not be expected to significantly alter the interaction of butanedione with these latter three amino acids, whereas borate has been specifically implicated in stabilization of an initially formed adduct of butanedione and arginine (Riordan, 1973). Thus, there is significantly indirect evidence that the modification in borate buffer arises from reaction only with arginine residues. Kinetic studies of the inactivation of L-lactate monooxygenase with butanedione in borate buffer revealed some expected complexity, which is discussed in detail below.

Although butanedione has been used most often in borate buffer for the inactivation of enzymes, other buffers have also been used (Huang & Tang, 1972; Pal & Coleman, 1976; Ehrlich & Coleman, 1977). L-Lactate monooxygenase was inactivated by butanedione in 2,6-lutidine buffer but not in phosphate or in imidazole buffers. The inactivation was a simple second-order reaction of enzyme and inactivator, and it was considerably faster than the inactivation in borate buffer. Exchange into phosphate buffer and removal of excess inactivator did not lead to any recovery of activity. This irreversibility may be a consequence of rearrangement of an initially formed dihydroxyimidazolidine derivative, which can dissociate to butanedione and arginine, or proceed to an irreversible complex (Riordan, 1973). If the latter is the case, it is important to note that the formation of the dihydroxyimidazolidine derivative and its rearrangement in lutidine buffer are considerably faster than the formation and stabilization of this derivative by borate in borate buffer. It has already been pointed out that the buffer employed has a significant effect on the reactivity of 1,2-dicarbonyl compounds with arginine residues (Cheung & Fonda, 1979). The results with L-lactate monooxygenase also indicate the buffer chosen for studies of inactivation of enzymes with butanedione has a very important influence on the results obtained and that several buffer systems should be examined to arrive at optimum conditions for inactivation.

Phenylglyoxal was also found to inactivate L-lactate monooxygenase in lutidine buffer. The rate of inactivation increased as the pH was raised. There was no recovery of activity on removal of excess inactivator and exchange into phosphate buffer. With [^{14}C]phenylglyoxal, it was found that complete loss of activity was correlated with the incorporation of 2 equiv of phenylglyoxal. On the basis of the work of Takahaski (1968), it has usually been assumed that phenylglyoxal reacts with arginine to give a complex with a 2:1 ratio of phenylglyoxal to arginine. However, there have been several examples of the inactivation of enzymes with phenylglyoxal in which a 1:1 stoichiometry has been found (Borders & Riordan, 1975; Werber et al., 1975; Berghauser, 1977; Philips et al., 1978, 1979). Consequently, it is not proper to assume a stoichiometry for the formation of the phenylglyoxal/arginine complex without experimental confirmation. In the case of L-lactate monooxygenase, amino acid analysis of the inactivated enzyme indicated that a single arginine residue was lost and that the lysine and histidine content was not changed. Because of the large number of arginyl residues in the enzyme (26 per subunit), care had to be taken in order to distinguish

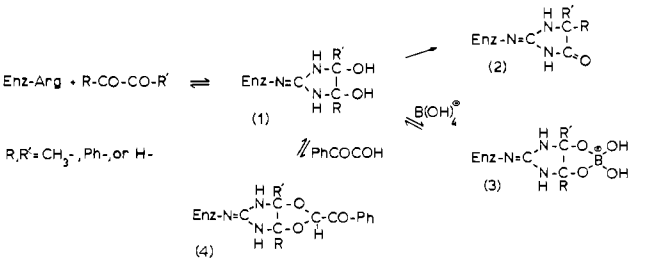
reliably between the loss of one and two residues of arginine by amino acid analysis. It was shown by using standard solutions that a 4% relative difference in arginine concentration could be reproducibly and reliably determined. The presence of one catalytically essential arginine in the enzyme can be confirmed by analysis of the data in Figure 4 by the method of Tsou (1962). There are certain limitations to the method (Rakitzis, 1978), and it must be assumed that modification of the essential residue(s) causes complete inactivation. For cases where all residues modified react at the same rate, Paterson & Knowles (1972) have formulated eq 2 where p is the

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

number of groups which can be modified by the inactivating reagent, i is the number of these which are catalytically essential, and a is the fractional activity remaining when m groups have reacted. The equation will remain valid if p is the number of equivalents of modifying reagent which can be incorporated and m is the number incorporated when a fractional activity remains. The number of essential residues can be determined by the value of i which best fits a plot of $a^{1/i}$ vs. m. From Figure 4, this is clearly one essential arginine residue. The inactivation of L-lactate monooxygenase is thus highly specific in that only one of the 26 arginyl residues per enzyme subunit is modified. If the reactive arginine is involved in the binding of anions (which might include carboxylate-containing substrates) to the enzyme, its enhanced reactivity could be rationalized by a recent proposal by Patthy & Thész (1980) that the pK_a of arginyl residues of anion binding sites will be lower than that of other arginines and that the lowered pK_a leads to a hyperreactivity toward α-dicarbonyl reagents. In the presence of D-lactate, a competitive inhibitor of the enzyme, the rate of inactivation is slowed considerably. This observation is most easily explained if the arginine which is modified on inactivation of the enzyme is located in the active site. Of course, it is also possible that binding of a substrate or competitive inhibitor induces a conformational change which diminishes the reactivity of the target arginine located outside the active site. From the available data on L-lactate monooxygenases, there is no evidence for significant conformational changes on the binding of substrates or inhibitors, but the possibility cannot be excluded rigorously.

On inactivation with phenylglyoxal, the enzyme loses the ability to catalyze the oxidation of L-lactate and the reduction of the FMN coenzyme, which is the first chemical transformation in the mechanism. This first reaction involves the generation of a carbanion at the α position of the substrate lactate followed by transfer of two electrons of the flavin coenzyme (Walsh, 1978). It appears that the inactivation blocks a step before the initial proton removal because the enzyme fails to undergo flavin modification by reaction with 2-hydroxy-3-butynoate. Native L-lactate monooxygenases undergo rapid modification of the coenzyme on reaction with 2-hydroxy-3-butynoate by a process which has been propsed to result from removal of the α proton of the acetylenic inactivator (Ghisla et al., 1976; Cromartie & Walsh, 1975). Thus, the evidence suggests that binding of carboxylate-containing substrates has been blocked by the inactivation with phenylglyoxal. Further support for this suggestion comes from the failure of the modified enzyme to bind inorganic phosphate, which is known to bind in the immediate vicinity of the FMN coenzyme in the active site of the native enzyme (Lockridge et al., 1972). Formation of a flavin/bisulfite complex on addition of negatively charged bisulfite is also prevented by arginine modification. On the other hand, access of external reagents to the coenzyme is not completely blocked, for dithionite instantly reduces the FMN in both native and modified enzyme. It is reasonable to assign tentatively the role of counterion of the carboxylate moiety of substrates to the reactive arginine residue in L-lactate monooxygenase. In fact, Ghisla & Massey (1980) have speculated that an arginine would have this role. The essential lysine residue in L-lactate monooxygenase reported by Choong et al. (1978) may well be involved in stabilization of the negative charge developing at N(1) of the flavin coenzyme on the addition of substrate carbanions to the flavin at N(5) (Massey et al., 1979; Ghisla & Massey, 1980).

Scheme I



Kinetic studies of the inactivation of L-lactate monooxygenase with butanedione in borate buffer and with phenylglyoxal have revealed several unusual results. In 50 mM borate buffer, the inactivation with butanedione shows saturation kinetics with respect to increasing butanedione concentration. Study of the interaction of butanedione and borate by NMR confirmed the observations originally made by Riordan (1973) that borate and butanedione interact to form a complex which lowers the concentrations of butanedione and borate in solution. As judged by NMR, however, the magnitude of this interaction is insufficient to account for the observed saturation phenomena. Also, in 20 mM borate buffer, saturation kinetics for inactivation are not found. Instead, clean second-order kinetic behavior is observed. In lutidine buffer, the inactivation, which occurred more quickly than in borate buffer, also showed simple second-order kinetic behavior. The inactivation of the enzyme with phenylglyoxal in lutidine buffer is first order in enzyme but second order in phenylglyoxal. Only one arginine is modified so the inactivation, which results on the incorporation of 2 equiv of phenylglyoxal, involves the formation of a single 2:1 complex of phenylglyoxal and arginine.

Unusual kinetic results similar to those found in this work have been noted in a variety of previous studies, but this is the first instance in which saturation kinetics for inactivation of an enzyme with butanedione have been found at one concentration of borate buffer but not at a higher one. The kinetics of inactivation of L-lactate monooxygenase with butanedione and with phenylglyoxal can be understood as a consequence of the two-step nature of the reaction of the guanidinium group of arginine with butanedione in borate and with phenylglyoxal (Scheme I; Takahaski, 1968; Riordan, 1973). In the absence of borate buffer, butanedione is expected to react reversibly with arginine to give a dihydroxyimidazolidine derivative (**1**), which could undergo rearrangement leading to irreversible inactivation. The inactivation would be first order in enzyme and first order in butanedione regardless of whether formation or rearrangement of **1** is rate determining. In the presence of a sufficiently high concentration of borate, the initially formed adduct could be rapidly trapped as a borate complex (**3**), which would be stable only in the presence of excess borate. Formation of the initial adduct would be rate determining, and the inactivation would be first order in enzyme and first order in butanedione, as

observed experimentally in 200 mM borate buffer. As the borate concentration in the buffer is decreased, it is possible that formation of the borate adduct of **1** will eventually become the slow step. Steric hindrance in the active site of the enzyme due to the incorporation of the butanedione structure could be expected to cause a decrease in the normally rapid formation of the borate complex with the cis-hydroxyl groups of adduct **1** (Roy et al., 1957). Increasing the amount of butanedione in the inactivation solution eventually will convert all of the enzyme to complex **1**. But since the rate-determining step is addition of borate to this reversibly formed adduct, addition of still more butanedione cannot increase the rate of inactivation. Saturation kinetics will be observed not because of the formation of a noncovalent Michaelis complex but because of the reversible formation of a covalent adduct (**1**) before the rate-dtermining step for inactivation. With this approach, saturation kinetics at low borate concentration but not at high borate concentration are readily explained. A simple extension of this argument to inactivation of the enzyme by phenylglyoxal can explain why the inactivation is second order in phenylglyoxal although only one residue of arginine is modified. If the initial adduct (**1**) of the enzyme and phenylglyoxal is formed reversibly, the steric bulk of the phenylglyoxal moiety in the active site could cause the addition of the second equivalent of phenylglyoxal to be rate determining, leading to an inactivation which is second order in phenylglyoxal. The third-order kinetics for inactivation by phenylglyoxal can also be explained with the formation of the adduct **1** as the rate-determining step for inactivation if complex **1** retains enzymatic activity, which is lost only on formation of complex **4**. In view of examples in which 1 equiv of phenylglyoxal per arginine residue has been found to be sufficient for inactivation (Borders & Riordan, 1975; Werber et al., 1975; Berghauser, 1977; Philips et al., 1978, 1979), this latter explanation seems less likely. It appears that the active site of L-lactate monooxygenase is sufficiently open to accommodate an equivalent of butanedione or phenylglyoxal readily. Incorporation of one of these reagents makes the addition of a second species (borate or phenylglyoxal) much more difficult.

The view of inactivation of enzymes by butanedione and phenylglyoxal developed in this work may be applicable in other instances. The lack of proportionality between the rate of inactivation and butanedione concentrations observed for lactate dehydrogenase (Yang & Schwert, 1972) and for alcohol dehydrogenase (Lange et al., 1974) may be due in part to slow interaction of the initially formed adducts with borate rather than to formation of a noncovalent Michaelis complex prior to inactivation. The fact that phenylglyoxal inactivates some enzymes by formation of a 2:1 complex with arginine and others by formation of a 1:1 complex can be viewed as a reflection of the accessibility of the initially formed 1:1 adduct to a second equivalent of phenylglyoxal. In most cases, there is room to add a second equivalent of phenylglyoxal as a 2:1 complex is formed, but in other cases a second equivalent cannot add. L-Lactate monooxygenase appears to be an intermediate case: a second equivalent of phenylglyoxal does add, but slowly in the rate-determining step for inactivation. This viewpoint obviates the need for proposing different chemistry for phenylglyoxal inactivation of enzymes which form 2:1 phenylglyoxal/argining complexes than for those with 1:1 complexes.

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Quantitative Solid-Phase Edman Degradation for Evaluation of Extended Solid-Phase Peptide Synthesis†

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ABSTRACT: Quantitative solid-phase Edman degradation was used for the amino acid sequence analysis of synthetic peptidyl-resins prepared by the Merrifield solid-phase procedure. A model peptide, Ala-[^{3}H]Pro-Ala-Gly-Phe-Ala-Gly-, was synthesized on a solid support and was sequenced to measure the efficiency of the solid-phase sequencing protocol used. An average of 92% of the first four residues was removed from the peptidyl-resin as indicated by subtractive amino acid analysis. Quantitation of the radioactive proline residue at cycle 2 revealed that it was efficiently recovered both from the acid conversion procedure (99%) and also following high-pressure liquid chromatography of the phenylthiohydantoin (Pth) amino acid (88%). In order to facilitate identification and quantification of the side chain protected Pth amino acids, we prepared these derivatives and characterized them by high-pressure liquid chromatography. Thereafter, by use of solid-phase Edman degradation as an analytical procedure, the synthesis of residues 2–118 of the heavy-chain variable region (V_H) of a homogeneous rabbit antibody was undertaken. At 10–15-residue intervals during the solid-phase synthesis, samples of peptidyl-resin were removed from the synthesis vessel and sequenced. When gross synthetic errors caused by deletion of amino acid residues were detected, the solid-phase synthesis was terminated and restarted by using modified protocols. A 117-residue peptidyl-resin was prepared finally which possessed the desired amino acid sequence as indicated by a series of solid-phase Edman degradation experiments. In the final degradation experiment on the 117-residue peptidyl-resin, a 92% efficiency for the automatic Edman reaction was measured ([^{3}H]Leu, penultimate amino-terminal residue). We have found two advantages for the concurrent use of solid-phase Edman degradation during an extended solid-phase synthesis: (1) on the basis of the levels of error due to incomplete incorporation of amino acids, the solid-phase assembly could be terminated in favor of restarting the synthesis, hence avoiding further work on a defective product and (2) direct verification of incorporation of amino acids, which during acid hydrolysis are destroyed (Cys, Trp) or are deamidated (Asn, Gln), is possible by high-pressure liquid chromatography of the corresonding Pth derivatives.

The solid-phase method of peptide synthesis as introduced by Merrifield (1963) has enjoyed widespread use as evidenced by an increasing number of reports describing the synthesis of biologically active peptides [reviewed by Erickson & Merrifield (1976)]. The basic solid-phase concept has been extended to the synthesis of oligosaccharides (Frecht & Scheurch, 1971) and oligonucleotides (Chapman & Kleid, 1973). In solid-phase peptide synthesis, improvements of procedure, protected amino acid derivatives, and solid supports have facilitated the synthesis of well-characterized peptides containing up to 40 amino acid residues (Yamashiro & Li, 1973; Tregear et al., 1974; van Rietschoten et al., 1975; Coy et al., 1977). Briefly, this has been accomplished in the following way. The carboxy-terminal amino acid is first attached covalently to a solid-support, usually polystyrene beads. This is followed by the sequential coupling of protected amino acids to the resin-bound amino groups until the desired peptide has been assembled. The completely deprotected peptide is then obtained by treatment of the peptidyl-resin with a cleavage reagent, usually liquid hydrogen fluoride. The crude product must then be purified and characterized with respect to its chemical homogeneity before any biological activity can be measured.

In order to establish the structure–activity relationships among analogues of peptides containing more than 40 residues, it is necessary to improve methods of chemical synthesis. For the solid-phase synthesis of a long peptide, it is anticipated that improved analytical methods will lead to improved synthetic methods. In this report we evaluate solid-phase Edman degradation as one analytical method for extended solid-phase peptide synthesis. Our goals were to verify experimentally the sequence of the peptide which is being synthesized and to detect error sequences which arise during synthesis from either incomplete coupling or incomplete deprotection.

It has been recognized that the Edman degradation (Edman, 1957) which is used to determine the amino acid sequence of peptides should prove useful in evaluating the progress of the solid-phase synthesis (Niall et al., 1972; Fankhauser et al., 1974; Birr & Frank, 1975). However, early attempts were hampered by difficulties in adapting the degradative procedure for amino acid sequencing of synthetic peptidyl-resins (Niall et al., 1972) and, in particular, in quantifying the resulting

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