Role of Arginine in the Binding of Thiamin Pyrophosphate to *Escherichia coli* Pyruvate Oxidase†

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ABSTRACT: The mode of interaction between *Escherichia coli* pyruvate oxidase and its cofactor, thiamin pyrophosphate (TPP), was studied with the aid of arginine-directed reagents. The enzyme is rapidly inactivated by either phenylglyoxal or 2,3-butanedione, with the cofactor, TPP, offering partial protection against these reagents. The inactivation by phenylglyoxal was found to be reversible. Experiments with [7-^{14}C]phenylglyoxal showed that while several arginine residues react with this reagent, TPP can prevent the labeling of one such residue. Furthermore, inactivation by 2,3-butanedione is attended by at least a 100-fold decrease in affinity of the enzyme for TPP. These results suggest a direct role for arginine in the binding of the cofactor.

Pyruvate oxidase is a peripheral membrane enzyme from *Escherichia coli* that catalyzes the oxidative decarboxylation of pyruvate to yield acetate and CO_2 (Hager, 1957). Located on the inner surface of the cytoplasmic membrane, the enzyme donates electrons to the aerobic respiratory chain of *E. coli* (Hager, 1957; Shaw-Goldstein et al., 1978). The isolated enzyme is conveniently assayed in vitro with the aid of artificial electron acceptors such as ferricyanide. As observed with other membrane enzymes, the inclusion of any of a variety of lipids and detergents in the assay medium greatly stimulates the catalytic activity of the purified enzyme (Blake et al., 1978).

Pyruvate oxidase is a tetramer composed of identical 60 000-dalton subunits (O'Brien et al., 1976). Each of these subunits possesses a tightly bound flavin adenine dinucleotide (FAD)[1] coenzyme (Williams & Hager, 1966) and binds a second coenzyme, thiamin pyrophosphate (TPP), in the form of a divalent metal ion complex (TPP–M^{2+}) (O'Brien et al., 1977).

Whereas the pyrophosphate anion is a competitive inhibitor of pyruvate oxidase with respect to TPP (Poludniak, 1972), free thiamin has no inhibitory effect and apparently does not interact with the enzyme. However, in the Breslow mechanism for pyruvate decarboxylation by TPP-requiring enzymes (Breslow, 1958), only the thiazolium ring of the cofactor is directly involved. This suggests that the function of the negatively charged pyrophosphate moiety of the cofactor complex (TPP–M^{2+}) is to anchor the complex in its binding site, while the thiamin moiety is more intimately involved in the catalysis. Riordan et al. (1977) and Takahashi (1977) have presented evidence that arginine residues are engaged quite frequently in the binding of negatively charged substrates, especially phosphorylated substrates, to enzymes. It was therefore considered that an arginine residue might be involved in the binding of TPP to pyruvate oxidase.

The arginine residues of proteins can be selectively modified by the reagents phenylglyoxal and 2,3–butanedione (Riordan, 1979). These reagents were employed in a study of the role of arginine in pyruvate oxidase. In particular, the possible involvement of arginine residues in the binding of TPP to pyruvate oxidase was investigated.

Materials and Methods

The purification of pyruvate oxidase is described elsewhere (O'Brien et al., 1976). Enzymic activity was normally determined by the standard ferricyanide reductase assay (Russell et al., 1977), which involves a preincubation of the enzyme with a detergent activator. When phenylglyoxal inactivation of the enzyme was monitored, the detergent activator was omitted and the enzyme assayed within seconds after sampling. In this way the reversal of phenylglyoxal inactivation upon dilution could be minimized. All chemical modifications of pyruvate oxidase were performed at ambient temperature.

[7-^{14}C]Phenylglyoxal was prepared from [7-^{14}C]acetophenone (New England Nuclear) by the method of Riley & Gray (1943), with the omission of the vacuum distillation step. The purity of the product was determined by its elution on a Sephadex G-10 column (Pharmacia Fine Chemicals) compared to that of the unlabeled compound (Takahashi, 1968). No labeled impurities were detected. The specific radioactivity of the [7-^{14}C]phenylglyoxal used in labeling experiments was 24 mCi/mol.

Commercial 2,3-butanedione was vacuum distilled prior to use. Freshly distilled butanedione formed clear aqueous solutions in contrast to some commercial products. Aqueous solutions of the reagent were used immediately after their preparation.

Determination of [7-^{14}C]Phenylglyoxal Incorporation. Pyruvate oxidase was incubated with [7-^{14}C]phenylglyoxal (see Figure 5). At intervals, a 50-μL aliquot of the incubation mixture was diluted into 0.5 mL of 0.1 M sodium citrate buffer, pH 4.0, in a 1.5-mL polypropylene centrifuge tube. Immediately, a 0.5-mL volume of 10% trichloroacetic acid was added, which precipitated the protein gradually. The first dilution was intended to reduce the possibility of the incubation buffer being trapped in the protein precipitate, which would lead to erroneously high labeling ratios. The precipitate was collected by centrifugation and washed 3 times with 5% trichloroacetic acid. The precipitate was solubilized in 12 mL of Biofluor (New England Nuclear) scintillation solvent and introduced into a scintillation vial. A 1.0-mL volume of 0.1 M acetic acid was added before scintillation counting to negate

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[1] Abbreviations: TPP, thiamin pyrophosphate; FAD, flavin adenine dinucleotide.

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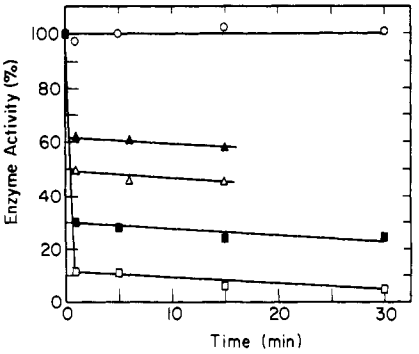

FIGURE 1: Concentration dependence of phenylglyoxal inactivation. Pyruvate oxidase was incubated at a concentration of 0.9 mg/mL in 0.1 M sodium phosphate–10 mM magnesium chloride, pH 7.8, and assayed before and after addition of phenylglyoxal to a concentration of 3.0 (▲), 5.0 (△), 10 (■), or 25 mM (□). Phenylglyoxal was not added to the control sample (○). No detergent activator was present in the assay medium (see Materials and Methods).

any possible variation in counting efficiency due to small amounts of water introduced with the protein precipitate. Counting efficiency was determined to be 83%.

An isotope dilution experiment showed that excess [7-^{14}C]phenylglyoxal was not trapped in the protein precipitate. The observed incorporation of [7-^{14}C]phenylglyoxal was greatly attenuated in the presence of excess "cold" phenylglyoxal, indicating that the observed labeling was due to specific binding of the reagent.

Determination of TPP Binding by Fluorescence Quenching. The binding of the cofactor TPP to pyruvate oxidase was monitored by a convenient fluorescence technique (O'Brien et al., 1977). The partial quenching of intrinsic tryptophan fluorescence observed in the presence of TPP is directly related to the binding of this cofactor by the enzyme. Thus the parameter $(F_0 - F)/(F_0 - F_s)$ may be identified with $\bar{\nu}$, the fractional saturation of cofactor binding sites. (Here F is the relative fluorescence intensity of the sample, F_0 is this value before addition of TPP, and F_s is this value in the presence of saturating TPP.) The TPP binding properties of the modified enzyme species were compared with those of the native enzyme by assuming that the tryptophan fluorescence of all enzyme species is quenched to the same extent upon binding of TPP.

Results

Reaction of Phenylglyoxal with Pyruvate Oxidase. The exposure of pyruvate oxidase to phenylglyoxal at pH 7.8 results in a biphasic inactivation of the enzyme (Figure 1). The extent of inactivation occurring during the more rapid phase increases with the concentration of phenylglyoxal. As the phenylglyoxal–arginine adduct has been reported to be somewhat labile, especially in buffers of high pH (Takahashi, 1968), the reversible binding of phenylglyoxal to one or more arginine residues was suspected.

Because the rate of the phenylglyoxal–arginine reaction decreases markedly with pH (Takahashi, 1968; Cheung & Fonda, 1979), the time course of the inactivation and the possibility of reversibility could be more readily investigated by using a medium of lower pH. At pH 6.0 the reaction again proceeds in two distinct phases, but the rapid phase of the inactivation becomes at least 1 order of magnitude slower (Figure 2). Pyruvate oxidase inactivated for 20 min under these conditions recovers more than half of its lost activity upon a 16-fold dilution into a pH 6.0 buffer free of phenylglyoxal (Figure 2). This implies that the observed inactivation is due at least in part to the reversible binding of phenylglyoxal to

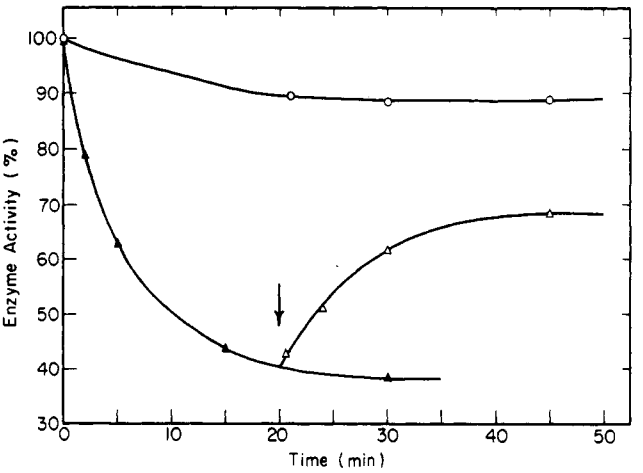

FIGURE 2: Reversal of phenylglyoxal inhibition by dilution. Pyruvate oxidase was incubated at a concentration of 0.21 mg/mL in 0.1 M sodium phosphate–5.0 mM phenylglyoxal, pH 6.0. After the indicated intervals, aliquots were assayed rapidly (▲) as described under Materials and Methods. A second enzyme sample was similarly treated with phenylglyoxal but after a 20-min incubation was diluted 16-fold into 0.125 M sodium phosphate, 12.5 mM magnesium chloride, 250 mM sodium pyruvate, and 125 μM TPP, pH 6.0. Aliquots (0.8 mL) of this solution were then assayed at intervals (△) by direct addition of sodium ferricyanide (0.2 mL of a 42.5 mM solution). A control was treated in a manner identical with that of the diluted sample except that no phenylglyoxal was added (○).

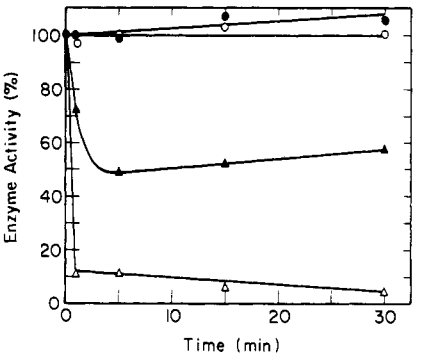

FIGURE 3: Thiamin pyrophosphate protection against phenylglyoxal inactivation. Pyruvate oxidase was treated as described in Figure 1. The incubation medium (0.1 M sodium phosphate–10 mM magnesium chloride, pH 7.8) contained 10 mM TPP (●), 25 mM phenylglyoxal (△), 25 mM phenylglyoxal plus 10 mM TPP (▲), or no additions (○).

pyruvate oxidase, probably in the form of a phenylglyoxal–arginine adduct.

The presence of a saturating amount of the cofactor TPP has a significant effect upon the phenylglyoxal inactivation of pyruvate oxidase occurring at pH 7.8 (Figure 3). The protection of the enzyme by TPP, albeit not complete, raises the possibility that an arginine residue(s) is (are) at the cofactor binding site. The failure of the cofactor to totally protect the enzyme indicates that at least one other arginine residue can react with phenylglyoxal even in the presence of TPP. Thus, the residues that react with phenylglyoxal can be tentatively divided into two distinct classes: (1) those residues that become inaccessible in the presence of TPP and (2) those residues that are accessible in both the presence and absence of TPP. Two corresponding types of inactivation, TPP sensitive and TPP insensitive, respectively, are therefore observed. The incomplete recovery of activity in the dilution experiment (Figure 2) is consistent with this assessment if it is assumed that the TPP-insensitive inactivation is not readily reversible. Also, if two independent modes of inactivation are assumed, the concentration dependence of the phenylglyoxal inactivation

Table I: Analysis of Observed Activities Assuming Two Independent Modes of Phenylglyoxal Inactivation

[PG][a] (mM)	A[b]	A_i[c]	A_s[d]	$1 - A_s$
3	0.62	0.93	0.67	0.33
5	0.49	0.88	0.56	0.44
10	0.30	0.81	0.37	0.63
25	0.12	0.63	0.19	0.81

[a] [PG] is the phenylglyoxal concentration employed. [b] A is the quasi-equilibrium level of activity. A is obtained by extrapolation of the nearly horizontal regions of the profiles in Figure 1 to time zero. [c] A_i is defined analogously to A but the plots extrapolated refer to the phenylglyoxal inactivation observed in the presence of 20 mM TPP. [d] A_s is determined from $A = A_i A_s$.

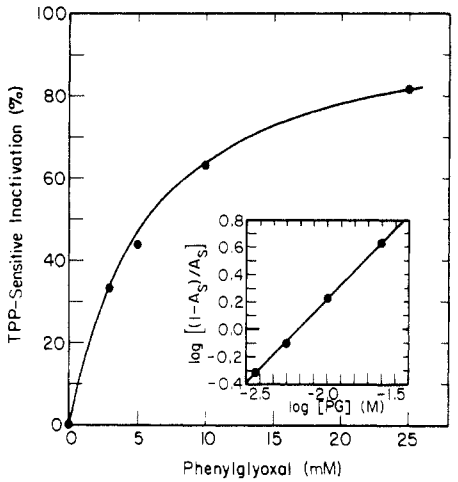

FIGURE 4: Concentration dependence of phenylglyoxal inactivation. The TPP-sensitive inactivation, $1 - A_s$, of pyruvate oxidase (see Table I and the text) is shown as a function of the phenylglyoxal concentration. The solid curve is a theoretical binding isotherm for a single site with K_d = 5.6 mM. The inset is a Hill plot of the data. The slope of the linear least-squares fit of the data is 1.0. The K_d was chosen to be 5.6 mM by a standard analysis of the Hill plot.

profiles can be readily understood.

Extrapolation of the nearly horizontal regions of the profiles in Figure 1 to time zero defines a quasi-equilibrium level of activity, A, for each phenylglyoxal concentration. (In what follows all activities are assumed to be normalized so that they range between 0 and 1.) If the enzyme is inactivated by two independent means, the observed activity, A, would be given by $A = A_s A_i$, where A_s and A_i are the activities that would be observed if each mode of inactivation, TPP sensitive and TPP insensitive, could be measured independently. Extrapolation of a set of profiles similar to those of Figure 1 but obtained with TPP-protected enzyme (data not shown) yields A_i as a function of phenylglyoxal concentration. The TPP-sensitive activity can then be derived from A and A_i according to $A = A_s A_i$. The different activities, A, A_i, and A_s, were determined in this manner for the several phenylglyoxal concentrations employed and are presented in Table I.

Figure 4 is a plot of the TPP-sensitive inactivation, $1 - A_s$, as a function of the phenylglyoxal concentration. The curve in Figure 4 is a theoretical binding isotherm for a single site:

$$\bar{\nu} = [PG]/(K_d + [PG]) \quad (1)$$

Here [PG] is the concentration of phenylglyoxal, K_d is the dissociation constant chosen to be 5.6 mM (see below), and $\bar{\nu}$ is the fractional saturation of the binding site. The good agreement between the inactivation data and the theoretical binding curve is consistent with the TPP-sensitive inactivation being a direct result of the reversible association of one phenylglyoxal molecule with one arginine residue. The inset of

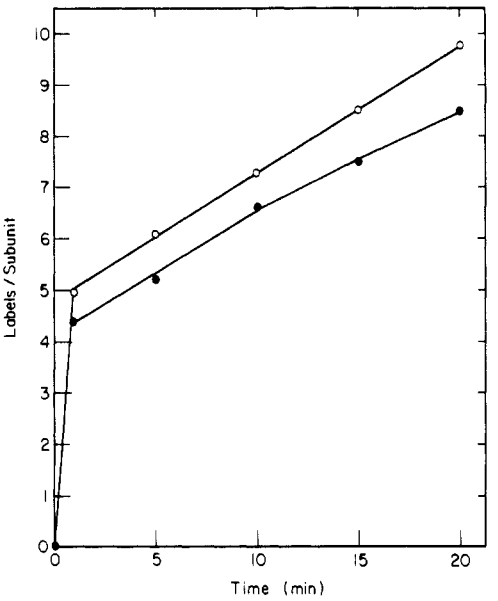

FIGURE 5: Incorporation of [7-^{14}C]phenylglyoxal into pyruvate oxidase. Pyruvate oxidase (3.0 mg/mL in 0.1 M sodium phosphate–10 mM magnesium chloride, pH 7.8) was treated with [7-^{14}C]phenylglyoxal (16.7 mM) and assayed at intervals for the incorporation of [7-^{14}C]phenylglyoxal (see Materials and Methods). One sample contained 1.0 mM TPP (●) and the other no TPP (○).

Figure 4 is a Hill plot of the data. The points fall on a straight line with slope 1.0, again in agreement with the single-site binding model. The dissociation constant, K_d, was determined to be 5.6 mM by a standard analysis of the Hill plot.

Phenylglyoxal binding to pyruvate oxidase was measured directly with [7-^{14}C]phenylglyoxal. It is critical to measure the amount of bound label in a manner which allows the trapping of the labile phenylglyoxal–arginine adducts. In the presence of 16.7 mM phenylglyoxal at pH 7.8 the enzyme rapidly incorporates about five reagent molecules per subunit (Figure 5). This labeling occurs during the fast phase of inactivation in Figure 1. When saturating TPP is present, this rapid labeling is reduced by 0.7 label/subunit (Figure 5). Thus the protective effect of TPP is correlated with a reduced labeling of the enzyme.

At lower phenylglyoxal concentrations (data not shown) the incorporation of labels occurring during the rapid phase of the reaction decreases. More importantly, the reduction of labeling observed in the presence of TPP also decreases. Thus, while a difference of 0.7 label/subunit was found with 16.7 mM phenylglyoxal, virtually no difference in labeling within experimental error was observed with 1.3 mM phenylglyoxal. The dependence of this labeling difference upon the phenylglyoxal concentration is roughly that predicted by the theoretical binding isotherm (Figure 4). The lability of the adduct makes it impossible to make a more quantitative correlation. The standard deviation in any given experiment was sufficient to detect labeling differences to within ±0.2 label, but there was variability between different experiments, due to the inability to efficiently and consistently trap all the labile adduct. In no experiment was there any indication of more than one phenylglyoxal being prevented from reacting with the oxidase in the presence of TPP. Apparently the TPP-sensitive inactivation is a direct result of the reversible binding of one phenylglyoxal molecule per enzyme monomer. In contrast, the TPP-insensitive inactivation is correlated with the binding of several reagent molecules per monomer.

Reaction of 2,3-Butanedione with Pyruvate Oxidase. A second arginine-directed reagent, 2,3-butanedione, also inac-

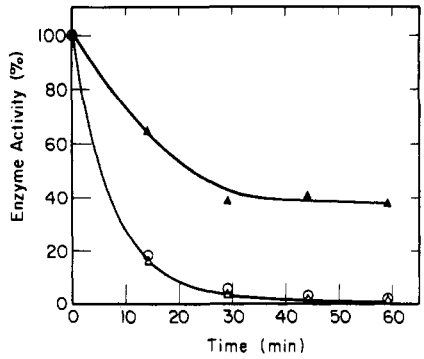

FIGURE 6: Inactivation of pyruvate oxidase by 2,3-butanedione and protection by TPP. Pyruvate oxidase was incubated at a concentration of 0.11 mg/mL in 50 mM 2,3-butanedione, 0.1 M barbital, 25 mM boric acid, and 10 mM magnesium chloride, pH 8.5, and assayed at the indicated times (△). A second enzyme sample was treated identically except that 10 mM TPP was included in the incubation medium (▲). The enzyme activities shown are corrected for the loss of activity in a control sample that contained no 2,3-butanedione or TPP. The inactivation occurring upon incubation in 50 mM 2,3-butanedione, 0.1 M barbital, and 10 mM magnesium chloride, pH 8.5, is shown for comparison (○).

tivates pyruvate oxidase. Figure 6 shows the time course of the inactivation that occurs when the enzyme is exposed to 50 mM butanedione in 0.1 M barbital, 10 mM magnesium chloride, and 25 mM boric acid at pH 8.5. The activity of enzyme inactivated by butanedione could be partially regenerated upon removal of the excess reagents, for example, by gel filtration in a butanedione- and borate-free buffer (data not shown). The long time required for activity regeneration (5 h) and the relative instability of pyruvate oxidase at high pH limited experimentation in this connection. No regeneration of activity is observed upon gel filtration in the presence of borate. The butanedione–arginine reaction reportedly becomes irreversible in the presence of borate (Riordan, 1973).

In the absence of borate, the time course and extent of the observed butanedione inactivation are not significantly changed (Figure 6). This indicates that if a ternary arginine–butanedione–borate adduct(s) is (are) formed in the presence of borate, the rate-limiting step is the combination of the arginine residue(s) with the butanedione molecule(s). This mechanism has been previously suggested (Riordan, 1973). Increasing the borate concentration above 25 mM only decreases the rate of the observed pyruvate oxidase inactivation (data not shown), presumably because of a butanedione–borate interaction.

Preincubation of pyruvate oxidase with the cofactor TPP at a saturating concentration results in a significant protection of the enzyme against butanedione inactivation (Figure 6). Again this protection is not nearly complete (compare Figure 3). This is consistent with the phenylglyoxal labeling experiments in which a large number of reactive residues were detected and the implication that only one of these residues is effectively blocked in the presence of TPP.

Binding of TPP to Pyruvate Oxidase Inactivated by 2,3-Butanedione. Because TPP partially protects pyruvate oxidase from inactivation by both phenylglyoxal and 2,3-butanedione and, at least in the case of phenylglyoxal, directly affects the binding of the reagent, it was considered that a reciprocal effect might be observed. Hence, the TPP binding properties of the inactivated enzyme were compared to those of the native enzyme. Because of the noted irreversibility of the butanedione reaction in the presence of borate, butanedione-inactivated enzyme was used in this investigation.

The binding of TPP to pyruvate oxidase was monitored by the convenient fluorescence quenching technique described

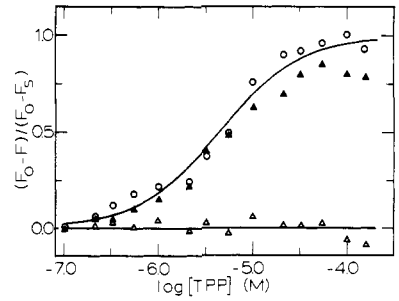

FIGURE 7: TPP binding to pyruvate oxidase as determined by fluorescence quenching (see Materials and Methods). The relative fluorescence intensity (F) of pyruvate oxidase solutions (23 μg/mL in 0.1 M sodium phosphate, 10 mM magnesium chloride, and 50 mM boric acid, pH 6.0) was monitored as aliquots of TPP solutions were added. F_0 and F_s are the fluorescence intensities of the native enzyme in the absence of TPP and in the presence of a saturating concentration of TPP, respectively. For the unmodified, native enzyme the parameter $(F_0 - F)/(F_0 - F_s)$ corresponds to the fractional saturation of TPP binding sites on the enzyme. The enzyme was previously incubated in 0.15 M barbital, 10 mM magnesium chloride, and 50 mM 2,3-butanedione, pH 8.5, in the presence (▲) or absence (△) of 10 mM TPP for 1 h. Excess reagent and TPP were first removed by gel filtration using a Sephadex G-25 column equilibrated and eluted with the titration buffer. A titration of native pyruvate oxidase under these conditions is shown for comparison (○).

under Materials and Methods. Three different pyruvate oxidase samples were studied: (1) native enzyme, (2) enzyme inactivated for 1 h in 50 mM 2,3-butanedione, pH 8.5, and (3) enzyme treated as in (2) but protected by the inclusion of TPP in the reaction medium. The butanedione-inactivated enzyme had less than 5% of its original activity, while the TPP-protected enzyme retained approximately 60% activity.

Figure 7 shows the apparent TPP binding isotherm of each enzyme sample. The native and TPP-protected species showed dissociation constants of approximately 5 μM, identical with the value obtained from equilibrium dialysis measurements with native enzyme under similar conditions (O'Brien et al., 1977). In contrast, the butanedione-inactivated enzyme apparently binds no TPP over the range of concentrations studied. Thus, the dissociation constant for TPP is probably increased at least 100-fold upon inactivation by this arginine reagent.

Discussion

In the presence of TPP the extent of inactivation of pyruvate oxidase by phenylglyoxal is markedly reduced, yet residual inactivation occurs (see Figure 3). Labeling studies employing [7-^{14}C]phenylglyoxal showed that while several residues per enzyme monomer react with this reagent, protection by the cofactor is attended by only a small reduction in labeling: approximately one residue per monomer (see Figure 5). Thus the reactive arginine residues of pyruvate oxidase were assumed to be distributed between two distinct classes: (1) those residues not accessible in the presence of TPP and (2) those residues accessible both in the presence and in the absence of TPP. Apparently the reaction of phenylglyoxal with residues of either class results in an inactivation of the enzyme. By comparison of the extents of inactivation observed in the presence and absence of TPP, the amount of inactivation due to the reaction of those residues protectable by TPP could be assessed (see Table I). This TPP-sensitive inactivation was found to increase with phenylglyoxal concentration in accordance with a simple reversible binding model (see Figure 4). The effect of this reagent is then similar to that of a competitive inhibitor, although the classical steady-state kinetics analysis could not be applied in this case because nonspecific inactivation also occurs. The only physically rea-

sonable model consistent with these data is that there is a single TPP-protectable arginine residue reacting with a single phenylglyoxal yielding an inactive enzyme adduct. For example, if two binding sites for phenylglyoxal were involved, one would have to assume, in order to fit the observed binding isotherm, that adduct formation at one site totally prevents binding at the second site and that binding to either site results in complete inactivation. The stoichiometry, measured with [7-^{14}C]phenylglyoxal, is consistent with only one site of interaction, although the correlation could not be quantitatively demonstrated. This is due to the lability of the adduct that must be trapped and the fact that the TPP-protectable labeling represents only 10–20% of the total labeling.

Employing 2,3-butanedione, a reagent that in the presence of borate reacts irreversibly with arginine, we could determine the effect of arginine modification upon the affinity of the enzyme for TPP. As in the case of phenylglyoxal, this reagent inactivates the enzyme in a manner only partially protectable by TPP (see Figure 6). Whereas the unprotected enzyme loses its high affinity for TPP upon butanedione exposure, TPP-protected enzyme retains a normal affinity for the cofactor after similar treatment (see Figure 7). The TPP-sensitive inactivation is therefore correlated with a loss of affinity for the cofactor.

Although the two diketone reagents used in this study are generally quite specific for arginine, exceptions have been noted in which these reagents have reacted with lysine residues or the terminal amino groups of peptides (Riordan, 1979). Reaction of diketones with these amines presumably involves Schiff base formation and a transamination (Takahashi, 1968), this latter transformation being irreversible in nature. As the phenylglyoxal inactivation of pyruvate oxidase is readily reversible, a transamination reaction is apparently not occurring here. Furthermore, the inactivation of the enzyme by 2,3-butanedione is irreversible in the presence of borate. As borate stabilization of butanedione–arginine adducts results from the combination of borate with a cis diol adduct (Riordan, 1973), it seems unlikely that borate would stabilize reaction products of butanedione with amino acids other than arginine. Thus, it is most probable that the TPP-sensitive inactivation of pyruvate oxidase by phenylglyoxal and 2,3-butanedione is due to their reaction with an arginine residue.

These findings indicate that an arginine residue is involved directly or indirectly in the binding of the cofactor, TPP, to pyruvate oxidase. The possibility of a favorable electrostatic interaction between the pyrophosphate moiety of the cofactor and a positively charged arginine residue makes the suggestion of a direct interaction attractive. The observation that the free pyrophosphate anion may be substituted for the cofactor in the protection experiments (data not shown) also lends credence to this view. An alternative explanation is that phenylglyoxal and 2,3-butanedione only indirectly affect cofactor binding via an allosteric mechanism.

While the reaction between phenylglyoxal and the arginine residues of proteins has been observed to be irreversible (Takahashi, 1968), there are many instances in which the adduct proved to be labile, especially in alkaline media (Philips et al., 1979; Daemen & Riordan, 1974; Kazarinoff & Snell, 1976). Furthermore, although the stoichiometry of the phenylglyoxal–arginine reaction product was originally reported to be 2:1, respectively (Takahashi, 1968), examples of proteins yielding products of 1:1 stoichiometry have been noted (Borders & Riordan, 1975; Mornet et al., 1979; Philips et al., 1978). Peters et al. (1981) have presented findings that suggest that the questions of irreversibility and stoichiometry are not unrelated. Results indicate that the formation of the stable 2:1 phenylglyoxal–arginine adduct proceeds via a 1:1 adduct that is formed reversibly (Philips et al., 1979; Mornet et al., 1979). It has been suggested that the formation of the 2:1 adduct is, in some cases, prohibited by the steric constraints of protein structure, accounting for the frequent observation of 1:1 adducts (Peters et al., 1981). The data presented here are consistent with this view. Apparently, at least one arginine residue of pyruvate oxidase forms a dissociable 1:1 complex with phenylglyoxal (K_d = 5.6 mM). The steric environment necessary to prevent the further conjugation of a 1:1 adduct with a second reagent molecule might well be provided in this case by a compact cofactor binding site.

Acknowledgments

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