Affinity Labeling of Pyruvate Kinase with 3,5-Dimethylpyrazole-1-carboxamidine†

Frank Davidoff,‡,* Stephen Carr, Michael Lanner, and Joel Leffler

ABSTRACT: In a rapid spectrophotometric assay, 3,5-dimethylpyrazole-1-carboxamidine reversibly inhibits rabbit muscle pyruvate kinase with a K_I of about 18 mM. Kinetic behavior of this reagent resembles Ca^{2+}, competing with Mn^{2+} and interacting with K^+ in a manner intermediate between competitive and noncompetitive inhibition. Prolonged incubation of pyruvate kinase with this carboxamidine leads to irreversible inactivation. This inactivation process appears to be pseudo first order; the inactivation rate is retarded by Mn^{2+}, Ca^{2+}, K^+, and NH_4^+ ions and increases with increasing pH. The ratio of inactivation rates at carboxamidine concentrations equal to $2K_I$ and K_I is about 1.2. Inactivated enzyme preparations exhibit a diminished V_{max} with respect to Mn^{2+}, but the apparent K_m is unchanged. During inactivation by dimethylpyrazolecarboxamidine a small amount of homoarginine appears in the enzyme; the differential increment in homoarginine formation associated with complete inactivation of unprotected enzyme exceeds that of enzyme protected with Mn^{2+} by 2 mol/237,000 g of protein. The data are consistent with affinity labeling of specific lysine residues in or near the divalent metal binding sites of pyruvate kinase.

Habeeb (1960) introduced 3,5-dimethylpyrazolecarboxamidine as a guanidinating reagent for proteins. In comparison with *O*-methylisourea and *S*-methylpseudourea, the compounds previously used for this purpose, the carboxamidine provided the advantages of greater stability in water and selective reaction with α- and ϵ-amino nitrogens of proteins at lower pH values, *i.e.*, below 10. The major structural features of 3,5-dimethylpyrazolecarboxamidine are a guanidinium and a hydrophobic ring of moderate bulk, which are very similar to the structures we have shown to be important for the binding of phenethylbiguanide and related compounds in or near the divalent metal site of rabbit muscle pyruvate kinase (Davidoff and Carr, 1972). We have therefore explored the possibility that dimethylpyrazolecarboxamidine could bind reversibly and selectively at the divalent ion site in pyruvate kinase in a rapid step, and then be subject to slower covalent attack by ϵ-amino nitrogens of lysine groups in the vicinity. This paper presents evidence that both of these steps occur; dimethylpyrazolecarboxamidine therefore meets most of the criteria for an affinity labeling reagent of good specificity (Baker, 1967; Singer, 1970).[1]

Materials and Methods

Rabbit muscle pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), in 2.5 M ammonium sulfate, was obtained from Boehringer Mannheim Corp., New York, N. Y., and separated from NH_4^+ by gel filtration (Davidoff and Carr, 1972). Polyacrylamide gel electrophoresis of several batches of this preparation at pH 8.0 or 9.5 revealed only a single component, both before and after repurification by the technique of Cottam *et al.* (1969). Pyruvate kinase activity was assayed spectrophotometrically (Davidoff and Carr, 1972, 1973a); in the current studies, pyruvate kinase was added last, and reaction rates were measured over a period of at least 5 min.

3,5-Dimethylpyrazolecarboxamidine as the nitrate was obtained from Aldrich Chemical Co., Milwaukee, Wis., or synthesized by the method of Scott and Reilly (1952); both preparations gave identical results. 3,5-Dimethylpyrazole and 3,5-dimethylpyrazolecarboxamide were obtained from the same commercial source, and all compounds were recrystallized from ethanol before use. Solutions of the carboxamidine reagent were adjusted to the desired pH with NaOH.

For analysis of homoarginine formation, pyruvate kinase was purified by reprecipitation from imidazole buffer (Cottam *et al.*, 1969). Following incubation, the enzyme was separated from dimethylpyrazolecarboxamidine either by precipitation and washing of the protein (Ray and Koshland, 1962) or by passing the incubation mixture over a column of Sephadex G-25 equilibrated with 10 mM NH_4HCO_3 buffer, pH 7.4. The protein content of the eluate was determined spectrophotometrically at 280 mμ, with correction for the anomalously low absorbance of the enzyme at this wavelength (Bücher and Pfleiderer, 1955). The protein was then lyophilized and hydrolyzed in 6 N HCl at 110° *in vacuo*, the HCl was removed, and aliquots were analyzed on a Spinco amino acid analyzer, Model 121. Arginine and homoarginine were quantitated by cutting out and weighing peaks using standards of authentic amino acids.

Results

Characteristics of Reversible Inhibition by Dimethylpyrazolecarboxamidine. When pyruvate kinase was assayed immediately upon addition to a standardized reaction mixture (Davidoff and Carr, 1972) containing 0.16 mM total Mn^{2+} and 40 mM K^+, pH 7.4, dimethylpyrazolecarboxamidine was found to be a moderately effective inhibitor of pyruvate kinase, 50% inhibition occurring at 36 mM under these conditions. In kinetic studies, dimethylpyrazolecarboxamidine was competitive with Mn^{2+}, while the kinetics observed with K^+

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[1] Some of this work was previously published in abstract form (Davidoff and Carr, 1973b).

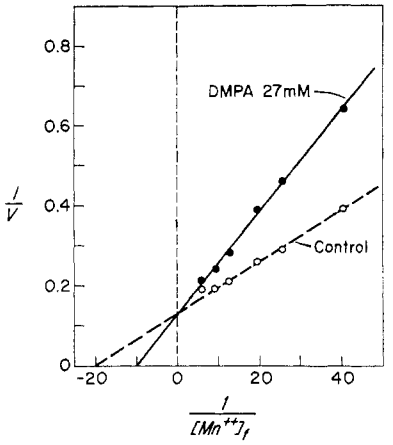

FIGURE 1: Kinetics of pyruvate kinase inhibition by dimethylpyrazolecarboxamidine. Enzyme activity was assayed spectrophotometrically in a volume of 1.5 ml; V is expressed in nanomoles of NADH formed per minute per cuvet. $[Mn^{2+}]_f$ was calculated as described previously (Davidoff and Carr, 1972). The abbreviation DMPA refers to dimethylpyrazolecarboxamidine.

were intermediate between competitive and noncompetitive (Figure 1). This kinetic behavior was identical with that of Ca^{2+} and of other guanidine-derived inhibitors of pyruvate kinase (Davidoff and Carr, 1972). By varying Mn^{2+} concentration at a series of different dimethylpyrazolecarboxamidine concentrations it was possible to obtain several values for K_p, the apparent K_m for Mn^{2+} at each inhibitor concentration (Dixon and Webb, 1964). When K_p was plotted as a function of inhibitor concentration according to Levenberg *et al.* (1957), the K_I for dimethylpyrazolecarboxamidine was determined to be about 18 mM (Figure 2). By direct assay, the activity of the lactate dehydrogenase used in the pyruvate kinase assay was unaffected by dimethylpyrazolecarboxamidine at concentrations up to 80 mM.

The related compounds, 3,5-dimethylpyrazole and 3,5-dimethylpyrazolecarboxamide, were weak inhibitors of pyruvate kinase, 33 mM inhibiting about 20%. Studies at higher concentrations were prevented by the limited solubility of these compounds.

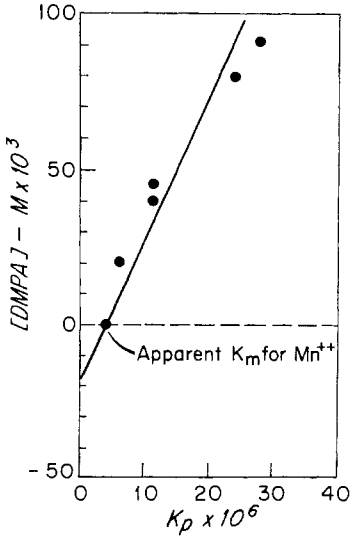

FIGURE 2: Determination of K_I of dimethylpyrazolecarboxamidine for pyruvate kinase. K_p (Dixon and Webb, 1964) was determined graphically from data similar to those in Figure 1.

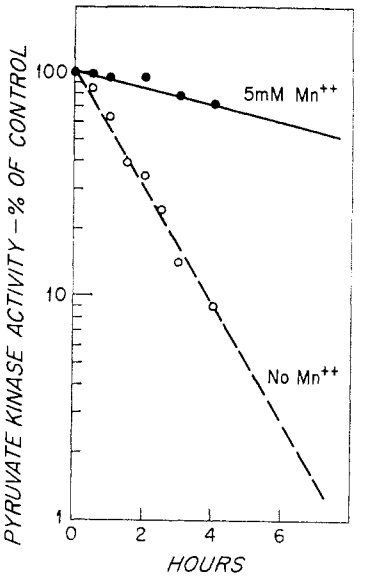

FIGURE 3: Irreversible inactivation of pyruvate kinase by dimethylpyrazolecarboxamidine. Pyruvate kinase at a concentration of 1.1 mg/ml was incubated at 20° in 0.5 ml total volume containing 25 mM Tris-HCl, pH 7.5, and either 160 mM tetramethylammonium chloride (control) or 160 mM carboxamidine; 5 mM $MnCl_2$ was included in control and carboxamidine samples where indicated. At increasing time intervals, aliquots of each sample were diluted and assayed as described in the text. Activity at each time point is expressed relative to its own control sample for each experimental condition.

Irreversible Inactivation of Pyruvate Kinase by Prolonged Incubation with Dimethylpyrazolecarboxamidine. KINETIC PARAMETERS. Irreversible inactivation of pyruvate kinase by dimethylpyrazolecarboxamidine was studied by incubating a relatively large amount of enzyme with a high concentration of the reagent (*e.g.*, 160 mM) in a small volume of solution. At increasing time intervals a small aliquot of pyruvate kinase was withdrawn and diluted successively into 0.05 M Tris buffer and then into the final assay cuvet containing 1 mM Mn^{2+} and 75 mM K^+ but no added inhibitor. The overall enzyme dilution was between 1500- and 6000-fold, thus bringing the concentration of dimethylpyrazolecarboxamidine in the assay well below that which inhibited reversibly. As is apparent from Figure 3, enzyme activity was lost progressively with time in a process which was linear on a semilog plot. The half-time of inactivation was about 1.2 hr with 160 mM dimethylpyrazolecarboxamidine at pH 7.5 and 20°, although there was some variation from experiment to experiment. At 0° and pH 7.5, 200 mM dimethylpyrazolecarboxamidine inactivated 50% of pyruvate kinase in 1.9 hr (average of two experiments).

PROTECTION BY DIVALENT METALS. One major criterion for affinity labeling is the ability of specific protector ligands to slow the rate of covalent reaction of the labeling reagent with the protein (Singer, 1970). From the kinetic data in Figure 1, all divalent metals which bind to specific sites on the enzyme would be expected to influence the extent of reversible dimethylpyrazolecarboxamidine binding, and hence to slow the rate of irreversible inactivation.

The activating divalent cation manganese, at a concentration of 5 mM, markedly slowed the rate of enzyme inactivation, about sevenfold under the conditions of Figure 3, but the order of the reaction appeared to be unaffected. Calcium, which inhibits competitively by displacing Mn^{2+} from the enzyme (Kachmar and Boyer, 1953; Davidoff and Carr,

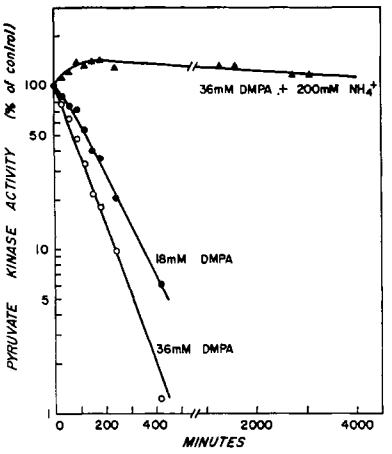

FIGURE 4: Inactivation of pyruvate kinase at concentrations of dimethylpyrazolecarboxamidine equal to K_I and $2K_I$; effect of NH_4^+. Incubation, dilution, and assay were carried out at a final pyruvate kinase concentration of 0.3 mg/ml in 50 mM Tris buffer at pH 8.0 with additions as indicated. Simultaneous control samples contained only 36 mM tetramethylammonium chloride.

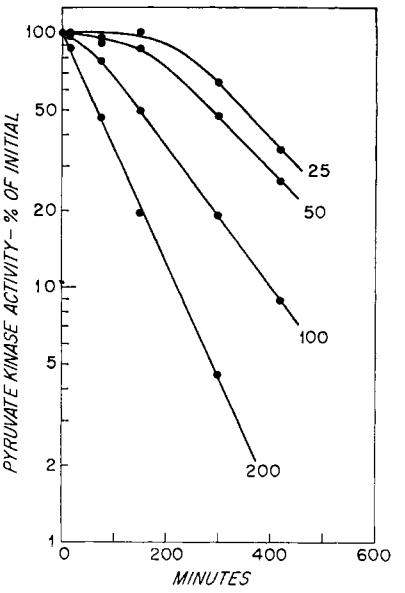

FIGURE 5: Inactivation of pyruvate kinase at increasing dimethylpyrazolecarboxamidine concentrations in the presence of K^+. The experiment was carried out as described in Figure 4; incubation samples contained 1.04 mg/ml of pyruvate kinase at pH 8.2, 75 mM K^+, and dimethylpyrazolecarboxamidine concentrations indicated beside each curve.

1972), also protected the enzyme from inactivation, the prolongation of 50% inactivation time ranging from 5- to 12-fold at a calcium concentration of 5 mM (data not shown).

PROTECTION BY UNIVALENT CATIONS. Certain univalent cations such as K^+, NH_4^+, and Rb^+ also activate muscle pyruvate kinase (Kachmar and Boyer, 1953), apparently through a mechanism other than change in divalent metal ion binding affinity of the enzyme (deAsúa *et al.*, 1970; Reuben and Cohn, 1970; Davidoff and Carr, 1972). Ammonium ions retarded the rate of inactivation by dimethylpyrazolecarboxamidine. Potassium also retarded inactivation, and on a molar basis, the two cations were equipotent (data not shown). Increasing univalent cation concentrations provided increasing protection and it was possible to prevent inactivation completely at high ratios of univalent cation to dimethylpyrazolecarboxamidine (Figure 4). To rule out the possibility that the protective effect of NH_4^+ could in part be attributable to chemical destruction of the carboxamidine, we took advantage of the specific ultraviolet (uv) absorbance maximum of the carboxamidine at 234 mμ (Habeeb, 1960). A solution of carboxamidine buffered at pH 8.4 and incubated with 150 mM NH_4^+ at 20° lost only about 15% of its absorbance at this wavelength in 24 hr, compared with 10% loss with 150 mM K^+ (both salts as the chloride), indicating that reagent degradation did not contribute significantly to the protective effect of either cation.

Pyruvate kinase has been observed to lose activity irreversibly when exposed for extended periods to Tris (Wilson *et al.*, 1967). We therefore explored the possibility that inactivation of the enzyme on incubation with dimethylpyrazolecarboxamidine might be due to potentiation of this Tris effect, rather than an independent action of the carboxamidine. Pyruvate kinase was therefore desalted on a Sephadex column equilibrated with 50 mM K^+ phosphate buffer, pH 7.4, and then incubated in the same K^+ buffer with 200 mM dimethylpyrazolecarboxamidine, thus avoiding contact with Tris entirely except for the final rapid dilution and assay. Inactivation was qualitatively the same with K^+ as when Tris buffer was used throughout, except, as discussed above, K^+ retarded the rate of inactivation.

EFFECT OF DIMETHYLPYRAZOLECARBOXAMIDINE CONCENTRATION ON INACTIVATION RATE. The rate of irreversible inactivation increased as the dimethylpyrazolecarboxamidine concentration was raised, but even at a concentration as low as 8 mM, 50% inactivation of the enzyme occurred at pH 8.0 in 75 min. The rate of a bimolecular reaction between a modifying reagent in solution and enzyme should change as a linear function of reagent concentration. In contrast, the increment in reaction rate between reagent bound to a specific site on the enzyme and functional groups on the protein should diminish progressively with increasing reagent concentration, the "rate-saturation effect" (Baker, 1967). The ratio of inactivation rates for dimethylpyrazolecarboxamidine concentrations equal to $2K_I$ and K_I was calculated from the linear portion of semilog inactivation plots similar to those shown in Figures 4 and 5 and was found to be 1.20 ± 0.15 ($n = 4$). This value is lower than the ratio of 2.0 expected for a bimolecular reaction, but does not differ significantly from the value of 1.3 associated with an affinity labeling process (Baker, 1967).

EFFECT OF PROTEIN CONCENTRATION ON IRREVERSIBLE INACTIVATION. When 200 mM dimethylpyrazolecarboxamidine was used in the incubation mixture, the semilog plot of enzyme activity loss was always linear. In many experiments at lower carboxamidine concentrations, however, a lag in the onset of inactivation became apparent (Figure 5), the duration of the lag being greater as the carboxamidine concentration was lowered. The variability of this lag from experiment to experiment initially suggested the possibility that variable quantities of NH_4^+ were carried through the Sephadex column in the desalting procedure, thus protecting the enzyme initially in a manner similar to the effect of added NH_4^+ or K^+ (see Figure 4). However, when the concentration of enzyme protein in the incubation mixture containing the carboxamidine was diminished below 0.3 mg of protein/ml the lag was completely eliminated, even at carboxamidine concentrations as low as 8 mM. Finally, when NH_4^+ or K^+ was added to these more dilute protein incubations the rate of inactivation was retarded without introducing a lag, thus excluding contamination with NH_4^+ from the Sephadex step as the explanation for the lag.

INFLUENCE OF pH ON INACTIVATION RATE. Guanidination of

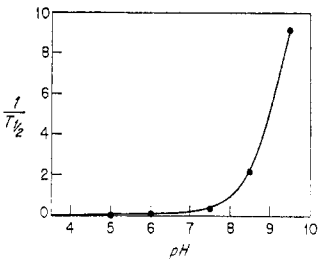

FIGURE 6: Effect of pH on inactivation rate of pyruvate kinase by dimethylpyrazolecarboxamidine. Incubations of pyruvate kinase with 200 mM tetramethylammonium chloride or dimethylpyrazolecarboxamidine were carried out as described in Figure 3 except that 20 mM K^+ was also present, and pH was adjusted with HCl or NaOH to the values indicated. $T_{1/2}$ refers to time required for 50% inactivation, in hours, determined graphically as in Figure 3. Each value plotted represents the mean of two separate experiments.

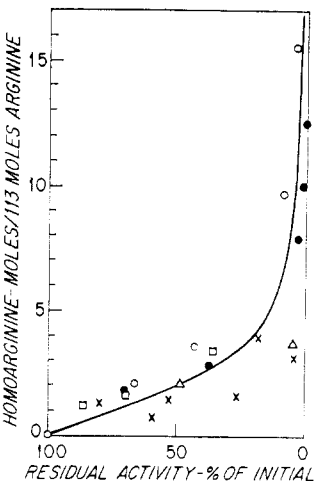

FIGURE 7: Conversion of lysine to homoarginine in pyruvate kinase incubated with dimethylpyrazolecarboxamidine. Each set of symbols represents one experiment in which incubations contained either 18, 50, or 200 mM dimethylpyrazolecarboxamidine at a pH of either 7.5 or 8.0. At increasing time intervals, samples were removed for dilution and assay, and immediately thereafter larger aliquots were taken for separation from the carboxamidine and hydrolysis. Circles, triangles, and X's represent samples subjected to trichloroacetic acid precipitation; squares are samples passed over Sephadex, as outlined in Materials and Methods. Enzyme activities below 3% of initial are estimates since measurement of activity became inaccurate at this level.

model compounds and of α- and ϵ-amino nitrogens in proteins by dimethylpyrazolecarboxamidine is a pH-dependent process (Habeeb, 1960; Hughes *et al.*, 1949), suggesting that the reaction involves attack by an unprotonated amino nitrogen on the N–C bond between pyrazole ring and amidine carbon. The rate of pyruvate kinase inactivation by the carboxamidine was strongly pH dependent, as demonstrated in Figure 6. The inactivation rate was apparently still increasing at pH 9.5, but higher pH values were not studied since the enzyme began to precipitate visibly in both controls and samples with dimethylpyrazolecarboxamidine as the pH was raised above 9. Over the pH range 6.5–9.5 the $T_{1/2}$ for inactivation by the carboxamidine decreased about 1000-fold, from ~100 to 0.1 hr. Since the pK_a of ϵ-amino nitrogens in proteins is 9.6–9.8 (Vallee and Wacker, 1970), while that of dimethylpyrazolecarboxamidine is 8.3 (Davidoff, F., and Carr, S., unpublished results), these data indicate that the nonprotonated form of the ϵ-amino nitrogen, the reagent, or both are involved in enzyme inactivation and support the occurrence of guanidination as the basis for the inactivation process.

KINETIC STUDIES DURING ENZYME INACTIVATION. For kinetic studies, multiple samples of enzyme were simultaneously diluted and assayed with increasing Mn^{2+} concentrations at each of several time intervals during incubation with the carboxamidine. As enzyme activity was lost progressively, the V_{max} decreased but the apparent K_m for Mn^{2+} remained unchanged, suggesting that irreversible loss of enzyme activity was not simply due to a decreased affinity of enzyme for Mn^{2+}.

During inactivation the enzyme solution, which was optically clear initially at pH values below 8, became progressively more opalescent with time. At concentrations of protein above 1 mg/ml, this opalescence was first noted at about 30% enzyme inactivation and increased so that as inactivation approached 90%, some flocculant precipitate accumulated at the bottom of the tube; at 0.2 mg of protein/ml, faint opalescence occurred only as inactivation was nearly complete. Opalescence and flocculation were markedly retarded when Mn^{2+}, Ca^{2+}, or NH_4^+ was present in parallel with retardation of inactivation.

Appearance of Homoarginine during Inactivation. Control samples of pyruvate kinase incubated with 200 mM tetramethylammonium chloride and then subjected to amino acid analysis contained no detectable homoarginine, even when the amino acid analyzer column was loaded to capacity. As enzymatic activity was lost during incubations with dimethylpyrazolecarboxamidine, increasing quantities of material with elution properties identical with authentic homoarginine standard were detected, and no other new peaks were noted on complete amino acid analysis. When homoarginine content is expressed as a function of residual activity, as shown in Figure 7, it is apparent that the number of lysine residues converted to homoarginine is a very small fraction of the total. Thus, using arginine as an internal standard and assuming the mole content of arginine to be 113 (Cottam *et al.*, 1969), 50% loss of enzyme activity was accompanied by formation of about 2 ± 1 mol of homoarginine/237,000 g of enzyme. This figure corresponds to modification of about 1.3% of the 148 lysines present per mol of enzyme. The individual experiments in Figure 7 were performed at two different pH values, 7.5 and 8.0, at various final protein concentrations ranging from 0.68 to 1.70 mg/ml, and at several carboxamidine concentrations from 18 to 200 mM. Consequently, the time required for 50% loss of activity varied from 560 to 70 min, and length of enzyme exposure to carboxamidine was very variable. Despite these wide differences in reaction conditions the data appear to cluster in relation to residual activity, indicating that the extent of initial homoarginine formation is related to residual enzyme activity rather than to time or pH.

Even in a true affinity labeling process, covalent modification of functional groups outside the specific site may be occurring more slowly elsewhere in the molecule, unless the specific site contains the only reactive amino acid in the protein or the reagent undergoes chemical activation only upon binding at the specific site (Singer, 1970). The extent of lysine conversion to homoarginine presented in Figure 7 must thus represent the upper limit of true site-specific guanidination of lysines. To eliminate the nonspecific guanidinations occurring outside the reagent binding site, enzyme at a concentration of 1.3 mg/ml was incubated with 18 mM dimethylpyrazolecarboxamidine without and with 5 mM $MnCl_2$; samples were taken simultaneously from both incubations, diluted, and

TABLE I: Differential Homoarginine Formation in Pyruvate Kinase.[a]

Time (min)	Manganese (5 mM)	Total Homoarginine Formed (mol/113 mol of Arg)	Δ Homoarginine Formed[b]	Fractional Act. Loss	Δ Fractional Act. Loss[b]	Δ Homoarginine Formed / Δ Fractional Act. Loss
570	+	1.2		0.17		
			0.8		0.34	2.35
	0	2.0		0.51		
1320	+	2.3		0.48		
			1.2		0.47	2.55
	0	3.5		0.95		

[a] Each sample was incubated in duplicate and amino acid analyses were repeated on all samples; values for homoarginine thus represent mean of four individual determinations. Values for fractional loss of activity are expressed relative to initial activity and represent mean of two samples. [b] Calculated as (value in absence of Mn^{2+}) − (value in presence of Mn^{2+}).

assayed for residual activity and hydrolyzed for determination of basic amino acids. The results, presented in Table I, indicate that the differential extent of homoarginine formation in the sample unprotected by Mn^{2+} exceeded that in the protected sample by 2.4 mol of homoarginine/237,000 g of protein at 100% enzyme inactivation.

Discussion

The interaction of dimethylpyrazolecarboxamidine with pyruvate kinase meets the major criteria for an affinity labeling process as set forth by Singer (1970) and Baker (1967) as follows.

(*1*) *Functional Evidence for Reversible and Site-Specific Binding of Modifying Reagent to Protein.* The carboxamidine binds reversibly at or near the divalent metal ion site, judging from the kinetic behavior with Mn^{2+} (Figure 1) and with K^+. Although the affinity of the carboxamidine for the enzyme is not extremely great (Figure 2), it is close to the value estimated for phenethylguanidine, a compound of similar size and hydrophobicity (Davidoff and Carr, 1972).

(*2*) *Diminution of Rate but not Extent of Covalent Modification of Protein by Specific Protector Ligands.* This effect was observed not only with manganese, a divalent activator of the enzyme (Figure 3), but also with a divalent inhibitor, calcium. Both K^+ and NH_4^+, which fulfill the absolute requirement of pyruvate kinase for an activating monovalent cation (Kachmar and Boyer, 1953), also protected the enzyme against inactivation. The mechanism by which monovalent cations activate the enzyme and partially antagonize reversible Ca^{2+} or guanidine derivative inhibition is not understood. The effect is probably not simply a function of altered enzyme affinities for Mn^{2+} *vs.* inhibitors since monovalent cations produce a "V" rather than a "K" effect kinetically (deAsúa *et al.*, 1970). Finally, direct measurement of divalent metal ion binding indicates that the changes induced by monovalent ions (Reuben and Cohn, 1970; Davidoff and Carr, 1972) cannot account for the activation of enzyme by monovalent ions. If, as suggested by this lack of influence on divalent ion binding, K^+ or NH_4^+ also does not influence the extent of dimethylpyrazolecarboxamidine binding, then these monovalent ions must modify the physical state of the protein in a manner which slows the rate of attack on bound carboxamidine by lysines, *e.g.*, by increasing the pK_a of specific ϵ-amino groups or their distance from the site of bound reagent, etc. The protective effect of monovalent cation thus appears to operate through a mechanism quite different from that of divalent cation.

(*3*) *Stoichiometric Modification of Catalytic Sites, i.e., Number of Moles of Label Should Equal Number of Catalytic Sites.* Pyruvate kinase has been shown to contain four Mn^{2+} binding sites (Reuben and Cohn, 1970; Davidoff and Carr, 1972) but appears to have only two substrate binding sites per mol wt 237,000 tetramer, each of which probably contains one of the divalent metal ion sites (Reynard *et al.*, 1961; Betts and Evans, 1968). The interpretation of the stoichiometry of homoarginine formation relative to numbers of divalent metal sites and catalytic centers is therefore not straightforward. At the minimum, however, the very small percentage of total lysines guanidinated (Figure 7) indicates that amino acid modification, if causally related to the associated activity loss, must be quite selective. Pyruvate kinase thus differs quite markedly from lactate dehydrogenase for which fractional activity loss exactly parallels the fraction of total lysines converted to homoarginine (Yang and Schwert, 1970).

The data for differential homoarginine formation in Table I are compatible with a total to two catalytic site specific lysines per 237,000 g of enzyme, and agree with the estimates cited above for the number of substrate binding sites per tetramer. The indication that total homoarginine formation is approximately twice this amount when the non-manganese-protected enzyme is 100% inactivated (Figure 7 and Table I) is also consistent with the modification of one lysine in or near each divalent metal ion site; however, all of these data were obtained close to the limits of sensitivity of the method, and more precise estimates must await the use of other techniques such as [^{14}C]amidine labeled dimethylpyrazolecarboxamidine.

(*4*) *Ratio of Rate of Active-Site Modification (K_3) to Rate of Modification of Residues Elsewhere on the Molecule (K_{M1}) Should be Large, Although the Specificity of the Reaction with Active-Site Residues Need Not Be Absolute.* The data discussed in the preceding section indicate that the small group of susceptible lysines of pyruvate kinase reacts with the carboxamidine much faster than lysines elsewhere in the molecule; when the incubation is prolonged, however, some of these other lysines also begin to react (Figure 7). This differential rate of susceptible lysine modification by the carboxamidine is not surprising when the rates of homoarginine formation in other proteins under the conditions used in most of these experiments are examined. Thus, at a concentration of 500

mM dimethylpyraxolecarboxamidine at pH 9.5, 3 hr were required for guanidination of 38% of the lysines in bovine serum albumin at room temperature; at pH 8.5, 500 mM carboxamidine guanidinated only about 6% of the lysines after 3 days at 0° (Habeeb, 1960). The reaction of dimethylpyrazolecarboxamidine with the specific lysines of pyruvate kinase thus appears to be at least 2500 times faster than the reaction rate with lysines in other proteins.

(5) *Enzyme Inactivation Should Exhibit a "Rate Saturation Effect" with Increasing Reagent Concentration.* As noted, the ratio of inactivation rates at dimethylpyrazolecarboxamidine concentrations equal to $2K_I$ and K_I was very close to that predicted for an affinity labeling process (Baker, 1967). However, it should be emphasized that this ratio of rates does not exclude a mechanism in which reversible binding of the reagent is required to permit a bimolecular reaction to occur elsewhere on the molecule.

It is not clear from these studies alone whether the mechanism of inactivation of pyruvate kinase after selective guanidination by dimethylpyrazolecarboxamidine involves elimination of binding of divalent metal to the enzyme or some step in the catalytic process beyond binding. Binding studies with modified enzyme, using radioactive Ca^{2+} or Mn^{2+}, may define the mechanism further. The involvement of lysine residues in transition metal binding to metalloproteins and enzyme–metal complexes has not been clearly established (Vallee and Wacker, 1970), although nitrogen-containing residues do form strong complexes with these ions (Darnall and Birnbaum, 1970). A role for the α-amino nitrogens of pyruvate kinase in divalent metal binding, and the possibility, therefore, that dimethylpyrazolecarboxamidine alters binding by their conversion to α-guanidino compounds, is rendered highly unlikely by the finding that all four N-terminal amino nitrogens are probably acetylated in the native enzyme (Cottam *et al.*, 1969). Evidence has been presented, however, for participation of histidines in divalent ion binding by this enzyme (Davidoff and Carr, 1973a).

It appears that dimethylpyrazolecarboxamidine, particularly labeled in the amidine carbon with ^{14}C, may be a useful reagent to identify and characterize that portion of the pyruvate kinase molecule in or near the divalent metal binding site; this techinique may also prove applicable to similar studies with other divalent metal ion dependent enzymes or divalent metal binding proteins.

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