The Acetylation of Creatine Phosphokinase with *p*-Nitrophenyl Acetate*

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ABSTRACT: The reaction of *p*-nitrophenyl acetate with creatine phosphokinase has been reported to inhibit this enzyme (Watts, D. C., Robin, B. R., and Crook, E. M. (1962), *Biochem J. 82*, 412). In view of earlier demonstrations that this reagent inhibits chymotrypsin and glyceraldehyde 3-phosphate dehydrogenase by a specific acetylation of the active site, it was of interest to compare in detail the reaction of *p*-nitrophenyl acetate with creatine phosphokinase and with these other enzymes.

Inhibition was shown to result from acetylation of the protein, but several characteristics of the reaction suggest that it is quite different from those observed with the other enzymes. Although creatine phosphokinase contains two sulfhydryl groups which react rapidly with iodoacetate (Mahowald *et al.*, 1962; Watts *et al.*, 1962) to yield an inactive enzyme, prior reaction with iodoacetate had no appreciable effect upon the acetylation of the enzyme with *p*-nitrophenyl acetate.

The limited acetylation by *p*-nitrophenyl acetate of a single serine residue of chymotrypsin (Hartley and Kilby, 1954; Balls and Wood, 1956; Oosterbaan *et al.*, 1962) and three cysteine residues of glyceraldehyde 3-phosphate dehydrogenase (Park *et al.*, 1961; Cunningham and Schepman, 1963) has proved of considerable interest with regard to the catalytic mechanisms of these enzymes. These specific reactions of activated amino acid residues are dependent upon the maintenance of the native conformation of the enzymes and result in the inhibition of enzymic activity. These reactive side-chain functions have been at least partly related to the chemical mechanisms of normal catalysis of these enzymes, leading to the suggestion that specific three-dimensional orientations of combinations of certain amino acid side chains produce centers of unusual chemical reactivity which participate in enzymatic catalysis (Cunningham, 1957; Cunningham and Schepman, 1963). Thus, it was of interest to determine if the inhibition of creatine phosphokinase (CPK)[1] (creatine–ATP transphosphorylase, ATP–creatine phosphotransferase, E.C. 2.7.32) by *p*-nitrophenyl acetate (Watts *et al.*, 1962) also resulted from the acetylation of activated amino acid residue(s) essential for maintenance of enzymic activity, and, if so, to compare the general properties of this acetylated enzyme with those of acetylchymotrypsin and acetylglyceraldehyde phosphate dehydrogenase.

Since creatine phosphokinase has been shown to have two essential "reactive" sulfhydryl groups (Watts *et al.*, 1962; Watts and Rabin, 1962; Mahowald *et al.*, 1962), it was of particular interest to determine whether the sites of reaction with *p*-nitrophenyl acetate were these same sulfhydryl groups, other sulfhydryl groups, or some other activated groups in the enzyme. The results of this investigation indicate that inhibition by *p*-nitrophenyl acetate results from acetylation of amino acid residues other than cysteine and thus occurs at a different site than that of reaction of iodoacetate.

Materials and Methods

Creatine Phosphokinase. Creatine phosphokinase was isolated from rabbit muscle according to procedure B of Kuby *et al.* (1954) as modified by Noda *et al.* (1960). Different preparations had specific activities of 50–75 units/mg when assayed by the phosphate method (Kuby *et al.*, 1954) and were homogeneous when examined in the ultracentrifuge. All preparations exhibited the same reactivity toward *p*-nitrophenyl acetate. Protein concentrations were determined spectrophotometrically at 280 mμ assuming $E^{1\%}_{1cm}$ 8.8 (Noda *et al.*, 1954; Kuby *et al.*, 1962). Routine assays for determination of the extent of inhibition were performed by the titration method of Mahowald *et al.* (1962).

Other Reagents. p-Nitrophenyl acetate was synthesized as described by Chattaway (1931). *p*-Nitrophenyl [^{14}C]acetate ($NO_2C_6H_4O^{14}COCH_3$) with a specific activity of 0.21 mc/mmole was prepared in this laboratory. Additional *p*-nitrophenyl [^{14}C]acetate with a specific activity of 1.11 mc/mmole and a sample of iodo[^{14}C]acetic acid ($ICH_2{}^{14}CO_2H$) with a specific activity of 2.04 mc/mmole were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals used were of reagent grade or the equivalent.

Inhibition with p-Nitrophenyl Acetate. The inhibition

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[1] Abbreviations used: CPK, creatine phosphokinase; ATP, adenosine triphosphate.

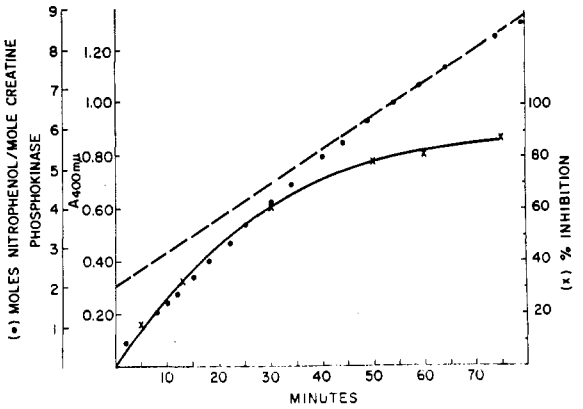

FIGURE 1: Comparison of the rate of inhibition of creatine phosphokinase by *p*-nitrophenyl acetate with the rate of liberation of *p*-nitrophenol; pH 7.0, 30°.

of creatine phosphokinase by *p*-nitrophenyl acetate was usually carried out at 30° in 0.1 M Tris–HCl buffer, pH 7.0, 3.3×10^{-6} M with respect to EDTA and 0.002 M in *p*-nitrophenyl acetate. In quantitative studies of the rate of inhibition, the enzyme concentrations used were near 1 mg/ml, while a higher concentration (4 mg/ml) was used when preparative quantities of the acetyl-enzyme were desired. [^{14}C]Acetyl-enzyme was isolated by the following procedure. After the desired reaction time, the inhibition mixture described above was added with vigorous stirring to a tenfold excess of an ice-cold mixture of acetone–ether–1 N HCl (20:5:1, v/v/v). The precipitate of acetyl-enzyme was collected by centrifugation and then washed at least three times with the acetone–ether–1 N HCl mixture to remove contaminating *p*-nitrophenyl acetate, *p*-nitrophenol, and acetate. It was then dried *in vacuo* over silica gel. In order to determine the extent of acetylation, a weighed quantity of the dried precipitate was dissolved by pepsin digestion (see below) and an aliquot of the digest was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Enzymic Digestions. PRONASE. Digestion of acetylcreatine phosphokinase (3.2 mg/ml) was carried out in 0.05 M Tris–0.015 M $CaCl_2$ (pH 7.8) using 0.4 mg of Pronase/ml (Pronase: *Streptomyces griseus* protease, B grade, 45000 P.U.K./g, Lot 502117, Calbiochem, Los Angeles, Calif.). The mixture was incubated under toluene at 37° for 24 hr with gentle agitation.

PEPSIN. Acetylcreatine phosphokinase (3.3 mg/ml) was hydrolyzed by 0.5 mg of pepsin/ml (crystallized two times, salt free, Lot 655, Worthington Biochemical Corp., Freehold, N. J.) in 0.05 N HCl. Incubation under toluene was carried out for 24 hr at 37°.

TRYPSIN. Acetylcreatine phosphokinase (1 mg/0.1 ml) was diluted with an equal volume of 0.12 M Tris buffer (pH 8.25), and 100–120 μg of trypsin/mg of acetylcreatine phosphokinase was added (trypsin crystallized two times, Worthington Biochemical Corp., Freehold, N. Y.). Incubation was for 24 hr at 37° under toluene.

Electrophoresis and Autoradiography of Peptides. High-voltage electrophoresis of protease digests was carried out on Whatman No. 1 chromatography paper in pH 3.6 pyridine–acetate buffer (pyridine–glacial acetic acid–water, 1:10:89, v/v/v) at 2000 v for 45–60 min. Radioactive peptides were located by autoradiography on Kodak (No-Screen) Medical X-ray film. Ninhydrin (3% in a solution of methanol–collidine, 95:5, v/v) was used to locate all peptides after electrophoresis.

Results

Inhibition by p-Nitrophenyl Acetate. The reaction of *p*-nitrophenyl acetate with proteins is conveniently followed as a function of nitrophenylate ion liberation by measuring increasing absorbance at 400 mμ (Hartley and Kilby, 1954). All of the reactions described here were carried out near pH 7.0 to minimize the spontaneous base-catalyzed hydrolysis of *p*-nitrophenyl acetate; all data have been corrected for this hydrolysis. The determination of nitrophenol concentration from measurements of optical density at 400 mμ was based on a standard curve made at the same pH at which the reaction with protein was carried out. Figure 1 compares the course of nitrophenylate liberation and loss of enzymic activity of creatine phosphokinase. A relatively rapid initial liberation of nitrophenol is followed by a somewhat slower continuing rate. The continuing rate may be extrapolated back to zero time and the number of moles of nitrophenol released in the initial reaction can then be estimated from the intercept. It may be seen that the inhibition of enzymic activity parallels the initial faster reaction in which approximately 2 moles of nitrophenol is liberated/mole of

TABLE I: Reaction of 0.002 M *p*-Nitrophenyl Acetate with Creatine Phosphokinase at pH 7.0 in 0.1 M Tris–HCl Buffer. Effect of Protein Concentration and Temperature.

CPK (μmoles × 10^2)	Temp (°C)	Initial Reaction of *p*-Nitrophenol[a]: CPK (mole: mole)	Secondary Reaction (rate) of *p*-Nitrophenol[a]: CPK/min (mole: mole/min)
0.95	25	2.1	0.11
0.99	25	2.0	0.14
1.64	25	2.1	0.11
3.16	25	1.6[b]	0.09
1.72	9–10	2.1	0.06
2.2	9–10	2.4	0.05

[a] *p*-Nitrophenol measured at 400 mμ. [b] *p*-Nitrophenol measured at 440 mμ.

TABLE II: Reaction of 0.002 M *p*-Nitrophenyl Acetate with Creatine Phosphokinase at pH 7.0 in 0.1 M Tris–HCl Buffer. Effect of Prereaction with 0.5 mM Iodoacetate, 1 Hr, 25°.

Iodoacetate	Creatine Phosphokinase (μmoles $\times 10^2$)	Temp (°C)	Initial Reaction of *p*-Nitrophenol[a]: CPK (mole:mole)	Secondary Reaction (rate) of *p*-Nitrophenol: CPK/min (mole:mole/min)
−	1.4	25	2.5	0.12
+	1.4	25	2.1	0.10
−	1.72	9–10	2.1	0.06
+	1.69	9–10	2.1	0.06
−	2.2	9	2.4	0.05
+	2.1	9	2.3	0.06

[a] *p*-Nitrophenol measured at 400 mμ.

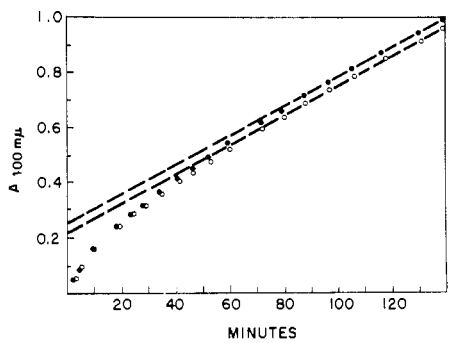

FIGURE 2: A comparison of the rates of liberation of *p*-nitrophenol from *p*-nitrophenyl acetate in the presence of creatine phosphokinase (●) and iodoacetate-inhibited creatine phosphokinase (○); pH 7.0, 25°. See the text for additional details.

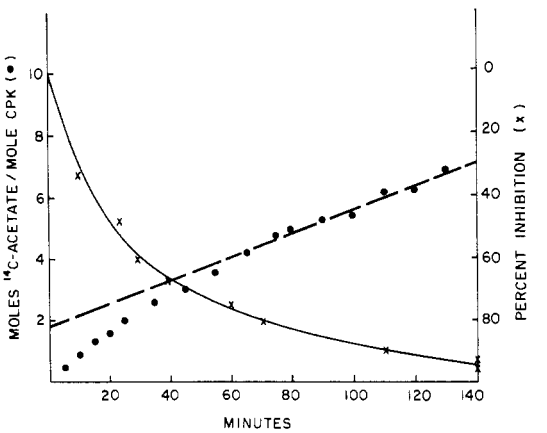

FIGURE 3: A comparison of the rate of incorporation of [^{14}C]acetate into creatine phosphokinase with the extent of inhibition of this enzyme in the presence of *p*-nitrophenyl [^{14}C]acetate; pH 7.0, 25°.

creatine phosphokinase. Although more detailed analyses of such biphasic reactions are possible, the experimental limitations of relatively slow rates and relatively high extent of hydrolysis of the *p*-nitrophenyl acetate over the course of this particular reaction are such that the usefulness of an analysis more complex than this simple extrapolation is doubtful. The data in Table I confirm the stoichiometry of the initial reaction over a threefold change in enzyme concentration. A decrease in the continuing rate of hydrolysis is found, as expected, when the reaction is carried out at lowered temperatures. Since a portion of the release of *p*-nitrophenol is the result of the acetylation of the protein which occurs continuously though at a declining rate over the longest time periods we have examined (see below), it is to be expected that this simple extrapolation procedure will yield higher values for the initial liberation of nitrophenol if times beyond 80–100 min at pH 7 and 30° are considered. The data of Tables I and II were obtained by extrapolation over less than 100 min since enzyme inhibition is two-thirds complete after only 30 min (Figure 1). Even after much longer periods of reaction, however, extrapolation to zero time indicated an initial rapid liberation of no more than 6 moles of *p*-nitrophenol/mole of enzyme. Under similar conditions Watts (1963) found that 14–16 moles of *p*-nitrophenol was liberated in the initial reaction. No explanation for this discrepancy is apparent at this time.

Effect of Previous Treatment with Iodoacetic Acid upon the p-Nitrophenyl Acetate Reaction. Since two of the six sulfhydryl groups of creatine phosphokinase have been shown to react with iodoacetic acid with a resultant loss of enzymic activity (Mahowald *et al.*, 1962; Watts *et al.*, 1962) and a sulfhydryl group has been shown to be the site of acetylation by *p*-nitrophenyl acetate in glyceraldehyde 3-phosphate dehydrogenase (Cunningham and Schepman, 1963), it was of interest to study the effect of prior iodoacetic acid

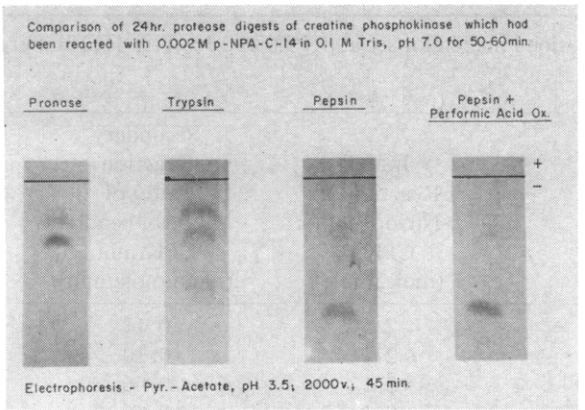

FIGURE 4: Autoradiograms of the [^{14}C]acetyl peptides resolved by paper electrophoresis at pH 3.6 (pyridine–acetate buffer) of several protease digests of [^{14}C]-acetylcreatine phosphokinase. This figure includes, for comparison, an autoradiogram of the [^{14}C]acetyl peptides which remain after performic acid oxidation of the pepsin digest of [^{14}C]acetylcreatine phosphokinase.

treatment upon the *p*-nitrophenyl acetate reaction with creatine phosphokinase. Figure 2 shows the liberation of *p*-nitrophenol to be only slightly, if at all, affected by prior reaction of the enzyme (3.4 mg/ml) with iodoacetic acid at pH 7.0, in 0.30 M Tris–HCl–10^{-5} M EDTA buffer containing 0.5 mM iodoacetic acid, for 67 min at 25°. At the end of the incubation period with iodoacetic acid less than 10% of the original enzymic activity remained. *p*-Nitrophenyl acetate was then added, diluting the sample to the usual conditions for the reaction with *p*-nitrophenyl acetate, and the release of nitrophenol was followed spectrophotometrically. The data from similar experiments are summarized in Table II and substantiate the conclusion that the reaction with iodoacetate has little effect on either the initial reaction with *p*-nitrophenyl acetate or the continuing hydrolysis rate.

Reaction with p-Nitrophenyl [^{14}C]*Acetate.* SPECIFICITY OF REACTION. In order to determine whether the reaction of creatine phosphokinase with *p*-nitrophenyl acetate did result in the formation of a stable acetyl-enzyme, the enzyme was treated with *p*-nitrophenyl [^{14}C]acetate. Reactions with *p*-nitrophenyl [^{14}C]-acetate were carried out under the same conditions as those described for the experiments listed in Table I. Assays for residual enzymic activity were performed directly on aliquots by the continuous titration technique. The reacted protein was obtained from the reaction mixture by precipitation with the acetone–ether–HCl mixture, digested with pepsin, and counted. Figure 3 shows that although approximately 2 moles of acetate are incorporated during the initial reaction period which corresponds to the loss of enzymic activity, additional incorporation occurs at a somewhat slower rate over the 2-hr reaction period.

Samples of acetylated creatine phosphokinase obtained after 40–50 min of reaction with *p*-nitrophenyl [^{14}C]acetate were digested with pepsin, Pronase, or trypsin. Electrophoresis and autoradiography of the resulting digests gave only a limited number of radioactive peptides, suggesting that acetylation occurred predominantly at a very few specific sites. The autoradiogram and peptide patterns reflected the specificity of the protease employed; representative autoradiograms are included in Figure 4.

PRETREATMENT WITH IODOACETIC ACID. Since treatment with iodoacetic acid had no effect upon the initial reaction of creatine phosphokinase with *p*-nitrophenyl acetate, it was of interest to determine whether there was an effect upon the incorporation of [^{14}C]acetate. The results in Table III are from an experiment in which the creatine phosphokinase (11.6 mg/ml) was incubated in 0.05 mM iodoacetic acid–0.33 M Tris–0.1 × 10^{-5} M EDTA (pH 7) at 30° for 1 hr (<5% enzymic activity remaining) before diluting with *p*-nitrophenyl [^{14}C]acetate to the usual reaction conditions. The control received no pretreatment with iodoacetic acid. Both preparations were isolated by precipitation with the acetone–ether–HCl mixture and counted after subsequent pepsin digestions. The data summarized in Table III suggest that pretreatment with iodoacetate has little effect upon the acetylation associated with loss of enzymic activity. This interpretation is confirmed by the observation (Figure 5) that prereaction with iodoacetic acid has no effect on the major Pronase, pepsin, and trypsin peptides which can be obtained by electrophoretic fractionation of the respective protease digests of [^{14}C]acetylcreatine phosphokinase. This finding indicates not only the independence of the sites of these two inhibitory reactions but also that the iodoacetate-reactive sulfhydryl groups are probably not near the *p*-nitrophenyl acetate reactive sites in the peptide chain since the addition of a carboxymethyl group would be expected to alter the mobilities of the [^{14}C]acetyl peptides produced by any of the proteases.

TABLE III: [^{14}C]Acetate Incorporation into Creatine Phosphokinase. Effect of Prereaction with Iodoacetic Acid.

Iodoacetic Acid	Moles of [^{14}C]Acetate/Mole of Protein Reaction Time with *p*-Nitrophenyl Acetate	
	40 min	120 min
–	3.9	7.6
+	3.6	7.2

Performic Acid Oxidation. Although the above results indicate that the two "active" sulfhydryl groups are not the site of acetylation by *p*-nitrophenyl acetate, the possibility of other sulfhydryl groups being the site of

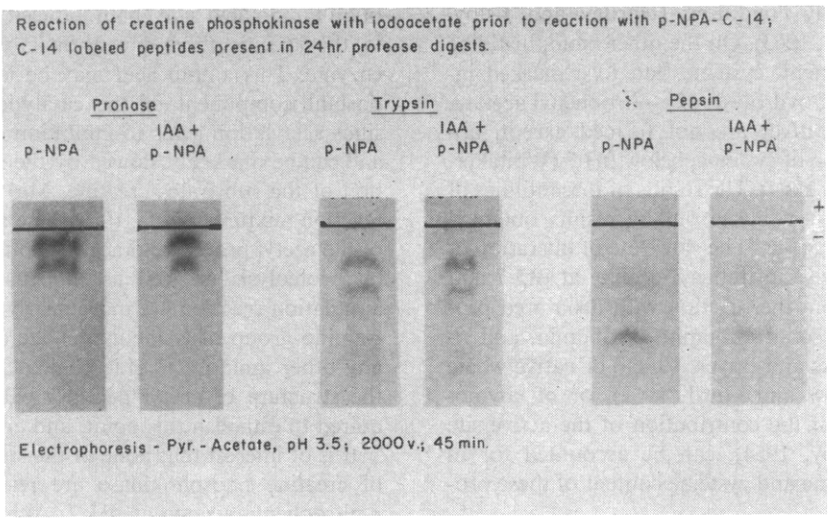

FIGURE 5: A comparison of the [^{14}C]acetyl peptides resolved by paper electrophoresis at pH 3.6 (pyridine–acetate buffer) of protease digests of [^{14}C]acetylcreatine phosphokinase and iodoacetate-pretreated [^{14}C]acetylcreatine phosphokinase.

acetylation was not ruled out. In order to resolve this question, aliquots of Pronase and pepsin digests and of a highly purified [^{14}C]acetyl peptide from a Pronase digest were subjected to performic acid oxidation (Hirs, 1956). After oxidation they were dried, redissolved, and counted. These experiments indicated that no more than 5–10% of the [^{14}C]acetate in the original digest was liberated by performic acid oxidation for 30 min at room temperature. Experiments on the isolated peptide resulting from Pronase digestion indicated no loss of radioactivity under these conditions. The small loss observed in the whole digests could be due to oxidation of small amounts of *S*-acetyl residues since Cunningham and Schepman (1963) have shown that performic oxidation of the *S*-acetyl residues of [^{14}C]-acetylglyceraldehyde 3-phosphate dehydrogenase leads to complete loss of all acetyl groups under these same conditions. The stability of the great majority of the [^{14}C]acetyl groups demonstrates that the site of the inhibitory acetylation of creatine phosphokinase is not a cysteine residue.

Figure 4 shows that this performic acid oxidation had no effect upon the electrophoretic mobility of the major [^{14}C] pepides produced by pepsin digestion of [^{14}C]acetylcreatine phosphokinase. Ascending paper chromatography in 1-butanol–glacial acetic acid–water (200:75:30) also indicated no change upon performic acid oxidation. Similar results were obtained with the [^{14}C] peptides obtained from Pronase digests. These results confirm that a sulfhydryl group is not the site of acetylation and that cysteine is most probably not a component of the peptide, since oxidation of a cysteinyl residue to cysteic acid would be expected to alter the electrophoretic mobility. It is likely that tryptophan, also, is excluded as a component of these peptides since performic acid oxidation produces a marked change in the chromatographic behavior of tryptophan in the solvent system used.

Discussion

The application of the reaction of *p*-nitrophenyl acetate with chymotrypsin to the study of the mechanism of action of this enzyme by a series of investigators beginning with Hartley and Kilby (1954) has led to the investigation of the usefulness of this reagent in the study of several other proteins and enzymes (Park *et al.*, 1961; Dirks and Boyer, 1951; Breslow and Gurd, 1962) including creatine phosphokinase (Watts *et al.*, 1962). The fundamental measurement of these studies was the increase of the rate of liberation of *p*-nitrophenol resulting from the addition of the protein to a solution of *p*-nitrophenyl acetate. Acceleration of the rate of *p*-nitrophenol liberation has been shown in the case of chymotrypsin and several other enzymes to be largely due to reaction with the active sites of these enzymes. The interpretation of the increased rate of *p*-nitrophenol liberation in the presence of any protein must, however, be based on a clear understanding of the nonspecific reactions of *p*-nitrophenyl acetate with certain amino acid residues which do not depend on the native structure of the proteins and are to a first approximation characteristic of the amino acid alone. Thus, it has been reported in studies of amino acids and simple peptides that little, if any, acetylation of α- and ϵ-amino groups occurs near pH 7, though it can be observed to occur slowly at higher pH values. Acetylation of the phenolic hydroxyl group of tyrosine derivatives was observed to occur at only about one-sixth the rate of *N*-acetylation, while acetylation of the hydroxyl groups of serine and threonine, or of the carboxyl groups of aspartic and glutamic acids, was not

observed under any conditions (Hartley and Kilby, 1954; Koltun *et al.*, 1963). On the other hand, histidine and, to a lesser extent, cysteine lead to a marked increase in the rate of hydrolysis of *p*-nitrophenyl acetate. Stable acetyl derivatives are not formed except, apparently, in the case of cysteine, below pH 7 (Whitaker, 1962; Ogilvie *et al.*, 1964). The results of these studies of model compounds are borne out by results obtained with a variety of proteins. The slow rate of liberation of *p*-nitrophenol from *p*-nitrophenyl acetate at pH 7 and below resulting from the reaction with denatured proteins [*e.g.*, bovine serum albumin, ovalbumin, and β-lactoglobulin (Dirks and Boyer, 1951)], of native whale myoglobin (Breslow and Gurd, 1962), or of chymotrypsin, exclusive of the contribution of the active site (Hartley and Kilby, 1954), can be accounted for in terms of the histidine and cysteine content of these proteins.

The catalysis of *p*-nitrophenyl acetate hydrolysis due to the active sites of chymotrypsin and of glyceraldehyde 3-phosphate dehydrogenase can be halted at an intermediate stage involving the formation of a specific and catalytically inactive monoacetyl enzyme (per monomer unit in the case of the dehydrogenase) (Hartley and Kilby, 1954; Balls and Wood, 1956; Park *et al.*, 1961), when the reaction is carried out at a somewhat lower pH. This inhibition of chymotrypsin and of glyceraldehyde 3-phosphate dehydrogenase by *p*-nitrophenyl acetate has been shown to result from the rapid acetylation of specific serine and cysteine residues in the respective enzymes (Oosterbaan *et al.*, 1962; Cunningham and Schepman, 1963). In both cases the acetylation appears to be closely related to an acylation which occurs in the normal catalytic operation of the enzymes upon their more typical substrates. Deacetylation, yielding fully active enzyme, can be readily induced by increasing the pH of the solution.

It is apparent from the evidence we have presented here, however, that the *p*-nitrophenyl acetate induced inhibition of creatine phosphokinase has quite different characteristics. Although inhibition has been shown to result from acetylation of a very limited number of sites in the enzyme, it can be seen in Figure 1 that this reaction is relatively slow and is partially masked by the very appreciable rates of continuing hydrolysis and acetylation at other sites. There is, then, no sharply defined "burst" of *p*-nitrophenol such as is observed with the other enzymes. It is, however, possible to relate the extent of acetylation, as measured with *p*-nitrophenyl [^{14}C]acetate, to the initial faster liberation of *p*-nitrophenol. Both measurements suggest that acetylation of as few as two specific sites causes inhibition of the enzyme. A second point of difference is the stability of this acetyl-enzyme. Neither chromatography of the acetylated protein on Sephadex G-25 nor exposure in aqueous solution to pH values from 7 to 9 leads to loss of these acetyl groups or to regeneration of catalytic activity. This suggests that, in the case of creatine phosphokinase, reaction with *p*-nitrophenyl acetate does not mimic in any direct fashion the normal catalytic function, but is, rather, the result of an increased reactivity of an amino acid side chain induced by its environment in the specific three-dimensional organization of the enzyme. This amino acid may be identified as an important component of the catalytic center, however, since acetylation leads to inhibition of enzymic activity and can be markedly slowed by the simultaneous addition of the substrates creatine, Mg^{2+}, and ATP to the reaction mixture (Watts, 1963). The chemical properties of the acetyl protein and acetyl peptides derived from it by proteolysis as well as the characteristics of the acetylation reaction are more nearly consistent with an ε-amino group of lysine as the site of acetylation than any other amino acid side chain. Chemical studies of the structure of acetyl peptides will, however, be required to establish this point, and are in progress.

It is of interest that none of the six sulfhydryl groups of creatine phosphokinase are readily acetylated by *p*-nitrophenyl acetate at pH 7. Although this is in accord with the slow and transient acetylation of cysteine itself at this pH observed by Whitaker (1962) and with the low reactivity of other sulfhydryl-containing proteins, ovalbumin and β-lactoglobulin (Dirks and Boyer, 1951), it should be noted that Watts and Rabin (1962) and Mahowald *et al.* (1962) have demonstrated that two of these six sulfhydryl groups undergo an unusually rapid reaction with iodoacetate which is pH independent over the range 6–9. Certain of the sulfhydryl groups of glyceraldehyde 3-phosphate dehydrogenase had been shown by Krimsky and Racker (1955) to be similarly unusually reactive toward iodoacetate, and it was subsequently shown (Cunningham and Schepman, 1963) that these same sulfhydryl groups are the site of the rapid and inhibitory acetylation of the dehydrogenase by *p*-nitrophenyl acetate. The relative activation of the sulfhydryl group in different enzymes toward iodoacetate, *p*-nitrophenyl acetate, and other reagents is clearly a complex phenomenon, reflecting in detail the chemical environment of this side chain, and affording, where additional information can be obtained, an insight into the catalytic mechanism of these enzymes.

Acknowledgments

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