Site of Action and Biphasic Effect of Neutral Salts in the Phosphorylase Kinase Reaction†

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ABSTRACT: The inhibition of phosphorylase kinase catalytic activity by 0.1 M neutral salts was predicted by the Hofmeister series of anions. The site of action of the salts was determined by the following evidence to be on the phosphorylase kinase molecule directly, rather than on its protein substrate. (1) Nonactivated kinase was more sensitive to salt inhibition than the activated form. (2) Ca^{2+} partially overcame the inhibition of nonactivated kinase. (3) Inhibition by Cl^- occurred with either phosphorylase or a tetradecapeptide containing the convertible seryl residue as substrate. (4) Phosphorylation of nonactivated phosphorylase kinase by protein kinase was markedly inhibited by $NaNO_3$, but this salt had little effect on the phosphorylation of histone by protein kinase. The influence of neutral salts on phosphorylase kinase activity was biphasic. Although activity was inhibited at low salt concentrations, it actually was stimulated as the salt concentration was increased. A similar biphasic response to various salt concentrations was observed in the velocities of autophosphorylation of phosphorylase kinase. The lag in the rate of product formation seen during the activity assay was less pronounced at inhibitory salt concentrations and was abolished at stimulatory salt concentrations. How the influence of salts relates to autophosphorylation and the lag is considered.

Phosphorylase *b* kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) is a key enzyme in the cascade of reactions associated with glycogenolysis and subsequent energy metabolism in skeletal muscle. Because low concentrations of Ca^{2+} are absolutely necessary for catalytic activity (Ozawa et al., 1967; Krebs et al., 1968), the enzyme often has been considered to be a hinge point coupling muscle contraction with energy production. Yet, despite the importance of this enzyme, few effectors of phosphorylase kinase have been described, and, other than Ca^{2+}, none has been shown to act predominantly on the phosphorylase kinase molecule, as opposed to its protein substrate.

Besides being phosphorylated by protein kinase, phosphorylase kinase also is able to phosphorylate itself (Walsh et al., 1971). This autophosphorylation reaction has been of continuing interest to us because of its probable involvement with the lag in the rate of product formation seen during the activity assay of nonphosphorylated kinase (Krebs et al., 1964; DeLange et al., 1968; Cohen, 1974; Carlson et al., 1975; Carlson and Graves, 1976). We wished to study how effectors of phosphorylase conversion would influence autophosphorylation and the lag. To do this, it was first necessary to find a class of effectors that would act predominantly on the phosphorylase kinase molecule, and not on its substrate.

There have been reports of the influence of neutral salts on phosphorylase kinase from rabbit skeletal muscle. Krebs et al. (1964) reported the activation of phosphorylase kinase in the presence of MgATP to be inhibited by NaCl and KCl. Sacktor et al. (1974) reported the inhibition of phosphorylase kinase catalytic activity by K^+, Na^+, and Li^+. In this work, we have studied in greater detail the inhibition of phosphorylase kinase catalytic activity by low concentrations of neutral salts. Evidence is presented that demonstrates that these salts act directly upon the phosphorylase kinase molecule. Correlations are made of the biphasic effects of the neutral salts on catalytic activity, autophosphorylation, and the lag.

Experimental Procedure

[γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (1964). Histone (type II-A) from calf thymus was purchased from Sigma. Neutral salts were purchased, when possible, in the anhydrous state. All the salts were dried in a vacuum oven at a pressure of less than 1 mmHg before use. NH_4Br, NH_4Cl, sodium acetate and potassium acetate were dried for 6 h at 50 °C. All other salts were heated at 95 °C for a minimum of 20 h.

All enzymes used in this work were isolated from rabbit skeletal muscle. The catalytic subunit of protein kinase, prepared by the method of Beavo et al. (1974), was generously provided by J. R. Skuster. Phosphorylase *b* was isolated as described (Fischer and Krebs, 1958). Residual AMP was removed by treatment with acid-washed Norit A. The glycerophosphate was removed by dialysis against 40 mM Hepes[1] or Tes, 30 mM mercaptoethanol, pH 6.8. Nonactivated phosphorylase kinase was prepared by the method of Brostrom et al. (1971) and, for most experiments, was further purified by DEAE-cellulose chromatography (Cohen, 1973; Jennissen and Heilmeyer, 1975). This kinase was stored frozen in 50 mM glycerophosphate, 2 mM EDTA, 1 mM dithioerythritol, pH 7.0. Phosphorylated kinase for use in gel filtration experiments and the experiment described in Figure 1 was prepared from nonactivated kinase that had not been subjected to DEAE-cellulose chromatography and that still contained a small

†From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011. *Received March 5, 1976.* Journal Paper J-8415 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 2120. This research was supported by a research grant (GM-09587) from the National Institutes of Health, United States Public Health Service, and the American Heart Association, AHA 72-621. A preliminary account of this work has been presented at the 4th International Congress of Metabolic Interconversion of Enzymes in Arad, Israel, 1975.

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tes, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; cAMP, 3′:5′-cyclic adenosine monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid; DEAE, diethylaminoethyl.

TABLE I: Inhibition of Nonactivated Phosphorylase Kinase by 0.1 M Neutral Salts at pH 7.5.[a]

Constant Ion	Ion Varied	% Inhibition
Na^+	acetate	+29 (stimulation)
	Cl^-	40
	SO_4^{2-}	64
	Br^-	77
	NO_3^-	88
	SCN^-	93
	I^-	95
Cl^-	Li+	37
	Na+	40
	Cs^+	44
	K^+	46
	NH_4^+	54
Br^-	Li+	64
	K^+	73
	$(CH_3CH_2)_4N^+$	73
	$(CH_3)_4N^+$	76
	Na+	77
	NH_4^+	80

[a] The assays contained phosphorylase *b* (8.6 mg/ml), buffer (50 mM Hepes, pH 7.5), MgATP (8.6 mM $Mg(CH_3CO_2)_2$, 2.5 mM [^{32}P]ATP), mercaptoethanol (13 mM), and salt (0.1 M) and were initiated by nonactivated phosphorylase kinase (0.34 μg/ml). After 30 min at 30 °C the incorporation of ^{32}P into phosphorylase was determined.

amount of endogenous protein kinase. The phosphorylation mixture contained nonactivated phosphorylase kinase (3.2 mg/ml), MgATP (16 mM Mg^{2+}, 6mM ATP), cAMP (4×10^{-5} M), and pH 6.8 glycerophosphate (40 mM). The phosphorylated kinase, which would be expected to be phosphorylated by both protein kinase and self-phosphorylation, was desalted on Sephadex G-25. It had a pH 6.8:8.2 activity ratio of 0.49. Phosphorylase and phosphorylase kinase concentrations were routinely determined spectrophotometrically by using absorbance indices of 13.0 (Kastenschmidt et al., 1968) and 12.0 (Hayakawa et al., 1973), respectively, for 1% protein solutions at 280 nm. Molecular weights of 1×10^5 for monomer phosphorylase (Cohen et al., 1971) and 1.28×10^6 for phosphorylase kinase (Cohen, 1973) were used for calculations of the extent of phosphorylation.

The phosphorylation of phosphorylase kinase, phosphorylase, and histone was determined by the ^{32}P incorporation into protein from [γ-^{32}P]ATP by utilizing the filter paper assay described by Reimann et al. (1971). For assays that measured the autophosphorylation of phosphorylase kinase, it was necessary to show that no significant phosphorylation occurred that could be attributed to cAMP-independent protein kinase activity. When nonactivated kinase was incubated with only MgATP (1 mM Mg^{2+}, 0.15 mM ATP) at pH 6.8 and 30 °C, less than 1 mol of phosphate was incorporated into the entire kinase molecule over a 45-min period. This concentration of MgATP is greater than the K_m of protein kinase for ATP (Reimann et al., 1971), but is below the K_m of phosphorylase kinase for ATP (Krebs et al., 1964). Inclusion of 10^{-5} M cAMP and 0.5 mM EGTA, which inhibits autophosphorylation (Walsh et al., 1971), still resulted in less than 1 mol of phosphate incorporation over the same period.

The tetradecapeptide was prepared synthetically on a Beckman 990 peptide synthesizer and purified to homogeneity

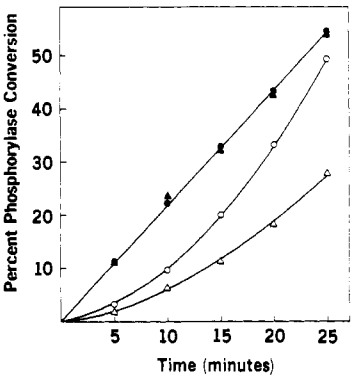

FIGURE 1: Effect of KCl on the activity of nonactivated and phosphorylated kinase. The reactions included phosphorylase (10.1 mg/ml), MgATP (6.9 mM $Mg(CH_3CO_2)_2$, 2.1 mM [^{32}P]ATP), buffer (47 mM Tes, pH 7.5), and mercaptoethanol (10.7 mM) and were initiated with phosphorylase kinase. Where included, the KCl concentration was 70 mM. Aliquots were removed at 5-min intervals for determination of ^{32}P incorporation. (●) Phosphorylated kinase (0.8 μg/ml); (▲) phosphorylated kinase plus KCl; (○) nonactivated kinase (2.5 μg/ml); (△) nonactivated kinase plus KCl.

by using combinations of gel filtration and ion-exchange chromatography. Purity of the peptide was determined by high-voltage electrophoresis and amino acid analysis. Formation of phosphorylated peptide was followed with an assay developed by G. W. Tessmer of this laboratory. Reaction aliquots (up to 20 μl) were applied to 1×2 cm strips of Whatman P81 phosphocellulose paper, which has a high affinity for the phosphorylated peptide. The reaction was stopped by dropping the paper strips into 1 N acetic acid. Excess [^{32}P]ATP was removed by washing the strips four times at 20 min each with the acetic acid. Each washing contained at least 10 ml of acid for each strip. The papers were then rinsed with ethanol, followed by ether, dried, and counted in 10 ml of Bray's solution.

Sedimentation velocity experiments were carried out in a Spinco Model E analytical ultracentrifuge. The rotor speed was 52 000 rpm, and the temperature was 22 °C. Pictures were taken at 4-min intervals with a Schlieren optical system.

Results

Inhibition of Activity by Low Concentrations of Neutral Salts. Preliminary studies with LiCl, KCl, NaCl, and NH_4Cl at a concentration of 30 mM showed that they all inhibit phosphorylase conversion by nonactivated kinase at pH 7.5 by about 25%. It was unclear if inhibition was actually due to the cations, the anion, or a general effect of neutral salts.

A detailed study of the relative effects of cations and anions on the conversion reaction was undertaken. The results of this study, in which 0.1 M salts were included in the assays at pH 7.5, are presented in Table I. When anions were varied and Na^+ held constant, there was a wide range of inhibition dependent upon the anion substituted. The inhibition ranged from 40% with chloride to 95% with iodide. The reaction actually was stimulated 29% with acetate. Therefore, the anions themselves influence the catalytic reaction regardless of the effect of the cation. With the exception of SO_4^{2-}, the pattern of inhibition obtained at pH 7.5 with these anions is closely predicted by the well-known Hofmeister series. The anionic Hofmeister series for increasing effectiveness in promoting protein destabilization has been reported by Lewin (1974) to be: $SO_4^{2-} < CH_3COO^- < Cl^- < Br^- < NO_3^- < I^- < SCN^-$.

Less inhibition was caused by chlorides than by bromides

when the anion was held constant and the cation varied (Table I), but the level of inhibition observed with either anion was relatively independent of the cation used. Note, for instance, that KBr and $(CH_3CH_2)_4NBr$ caused the same inhibition. The Li^+ and NH_4^+ cations were furthest from the norm and caused the least and the most inhibition, respectively, with both anions. This behavior of Li^+ and NH_4^+ follows the cationic Hofmeister series: $Li^+ > K^+ > Cs^+ > NH_4^+$ (Lewin, 1974). In summary, the conversion of phosphorylase by nonactivated phosphorylase kinase is influenced both by cations and by anions, but to a much greater extent by the latter. The results indicate that a locus exists on kinase, or phosphorylase, or both, that is sensitive to anions and that the effect follows the Hofmeister series.

During the activity assay of nonactivated phosphorylase kinase at neutrality there is a lag in the rate of product formation (Krebs et al., 1964); i.e., the enzyme evidently becomes more active with time. Inclusion of 0.1 M salt in the assay of nonactivated kinase at pH 6.8 altered this lag in the rate of product formation. Despite the fact that the most pronounced lag is normally seen at pH 6.8, inclusion of NaCl, NaBr, or $NaNO_3$ in the assay caused the progress curve to become more linear. Holding the concentration of $NaNO_3$ constant at 0.1 M and lowering the kinase concentration also caused the lag at pH 6.8 to become less pronounced. The process through which salts make the progress curve more linear is not totally clear. Any one of a number of observable changes occur in the progress curve when pH, salt, and enzyme concentrations are varied. The onset of the lag may be delayed, only the initial portion of the lag may occur, or the duration of the lag may be lengthened. It is often difficult to determine which of these is the predominant change because limiting substrate concentration and enzyme instability become factors with time. It is clear, however, that low concentrations of neutral salts do inhibit the autoactivation of nonactivated kinase.

Sodium nitrate was used as the neutral salt in many experiments because it inhibited kinase activity nearly as much as NaSCN or NaI, but was not believed as likely to cause irreversible inhibition. The reversibility of inhibition by 0.1 M $NaNO_3$ was tested by preincubating at pH 6.8 and assaying at pH 8.2, at which the enzyme is most active. This allowed a substantial dilution of the salt into the assay. Preincubation of nonactivated kinase (29 μg/ml) for 15 min at 30 °C with 0.1 M $NaNO_3$ followed by dilution to 0.7 mM $NaNO_3$ and 0.2 μg/ml kinase provided the same kinase activity as that obtained when kinase (0.2 μg/ml) was not challenged with any $NaNO_3$ except for 0.7 mM $NaNO_3$ in the assay. Therefore, the inhibition of nonactivated kinase by 0.1 M $NaNO_3$ is completely reversible.

Preincubation of kinase with phosphorylase, a process that stimulates kinase activity (Kim and Graves, 1973), was examined to see if it would alter the salt inhibition. The percentage inhibition brought about by 70 mM KCl at pH 7.5 was the same whether or not the kinase was preincubated with phosphorylase.

Site of Inhibition of Neutral Salts. Experiments were performed to determine if neutral salts inhibited the reaction by binding to the enzyme or to the substrate. Figure 1 shows the effect of 70 mM KCl on phosphorylase conversion at pH 7.5 by activated (phosphorylated) and nonactivated phosphorylase kinase. The activated kinase is not inhibited at this level of KCl (closed symbols), whereas the nonactivated kinase is (open symbols). This result suggests that the anion probably is acting directly on the kinase and not on the substrate, phosphorylase. If the major locus of action by the anion were on the substrate, then one would have expected inhibition to occur with both forms of kinase. In a separate experiment with 0.1 M $NaNO_3$, which is more inhibitory than KCl, the activated kinase also was inhibited. The inhibition of nonactivated kinase was, however, five times greater than the inhibition of activated kinase. So, again there was a differential effect of salts on the two forms of kinase.

Added evidence that the anion acts on the kinase molecule was obtained with experiments that measured the ability of Ca^{2+} to partially overcome salt inhibition. The 85% inhibition of nonactivated kinase at pH 7.5 by 70 mM KCl was reduced to 25% by addition of 10^{-4} M Ca^{2+}, a known activator of the kinase molecule (Ozawa et al., 1967; Krebs et al., 1968). Neither differential salt inhibition of nonactivated and activated kinase nor the ability of Ca^{2+} to overcome salt inhibition is absolute proof, however, that the salt acts on kinase. Both phosphorylation and addition of Ca^{2+} have been reported to increase the affinity of phosphorylase kinase for phosphorylase (Krebs et al., 1964; Heilmeyer and Haschke, 1972). If the salts acted on phosphorylase, causing it to be bound more poorly by kinase, then activation of the kinase or addition of Ca^{2+} would decrease the salt inhibition. Different types of experiments therefore were required to determine, without doubt, the site of salt action.

The phosphorylation of phosphorylase kinase by the catalytic subunit of protein kinase was also influenced by neutral salts. When nonactivated phosphorylase kinase was used as substrate, 0.1 M $NaNO_3$ inhibited its phosphorylation by protein kinase by approximately 50%. $NaNO_3$, however, caused only a slight inhibition (less than 10%) of the phosphorylation of histone by protein kinase. These results suggest that there is a locus of salt sensitivity on the phosphorylase kinase molecule.

More conclusive evidence that anionic inhibition of phosphorylase conversion is directed toward the phosphorylase kinase molecule would be to show that the effect still occurs with an alternative substrate for the kinase. A low-molecular-weight alternative substrate would be desirable because it would less likely be influenced by low concentrations of the neutral salts. A tetradecapeptide corresponding to residues 5–18 at the amino terminus of glycogen phosphorylase (Titani et al., 1975) and containing the convertible seryl residue has been shown to be a substrate for phosphorylase kinase (Nolan et al., 1964; Tessmer and Graves, 1973). This peptide, which has the sequence Ser·Asp·Gln·Glu·Lys·Arg·Lys·Gln·Ile·Ser·Val·Arg·Gly·Leu, was used as a substrate in experiments measuring the inhibition by neutral salts. It was found that 70 mM KCl inhibited peptide phosphorylation by nonactivated kinase at pH 7.5 by approximately 85% (Figure 2). This inhibition was partially overcome by addition of 10^{-4} M Ca^{2+} (results not shown). Because it is unlikely that this concentration of KCl could affect the peptide, the salt inhibition of peptide conversion was interpreted as being directed through the kinase molecule. The sum of the data in this section provides strong evidence that the inhibition of phosphorylase kinase activity by neutral salts is caused predominantly by anions acting directly on the kinase molecule.

Stimulation of Activity by High Concentrations of Neutral Salts. Whereas inclusion of 0.1 M salts in the kinase assay caused inhibition of catalytic activity, a second effect was found at greater salt concentrations. Nonactivated kinase was preincubated at pH 6.8 with various concentrations of $NaNO_3$. Assays of catalytic activity at pH 6.8 were then initiated with aliquots taken directly from the preincubation mixture and diluted threefold into the assay. Low concentrations of $NaNO_3$

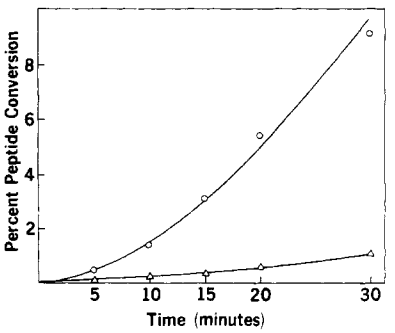

FIGURE 2: Effect of KCl on the phosphorylation of the tetradecapeptide. The reaction mixture included peptide (0.7 mM), MgATP (8.1 mM $Mg(CH_3CO_2)_2$, 2.5 mM [^{32}P]ATP), buffer (40 mM Hepes pH 7.7), and mercaptoethanol (4.3 mM) and was initiated with nonactivated kinase (4 μg/ml). Where included, the KCl concentration was 70 mM. Aliquots were removed at times indicated for measurement of ^{32}P incorporation into peptide. (○) H_2O; (△) KCl.

caused inhibition of the kinase activity, but, as the concentration in the preincubation mixture was increased above 0.15 M, the inhibition grew smaller (Figure 3). When the $NaNO_3$ concentration of the preincubation mixture reached 0.35 M, stimulation of activity was observed. So, there are two opposing effects of $NaNO_3$ on kinase activity at pH 6.8. It causes inhibition at low concentrations and stimulation at high concentrations.

At the highest level of $NaNO_3$ shown in Figure 3 (1.05 M in the preincubation mixture and 0.35 M in the assay), the activity at pH 6.8 was stimulated fivefold during the first 5 min of the assay. The insert of Figure 3 shows the time courses for the control reaction and for the reactions in which maximal stimulation and inhibition occurred. Note that no lag is observable in the time course corresponding to maximal stimulation. In all cases in which nitrate stimulation occurred, there was no observable lag. In the case of the time course corresponding to maximal inhibition, a lag became evident after the reaction had proceeded for 30 min.

Stimulation of nonactivated kinase at pH 6.8 by high salt concentrations was not a specific effect of $NaNO_3$. RbBr, CsBr, and LiBr were progressively more effective than $NaNO_3$ in causing stimulation when preincubated for 30 min at 1.2 M with the kinase. Activation was also observed if the salts were diluted to 10 mM in the assay after the initial preincubation was carried out at salt concentrations of 1.2 M. After 10-min preincubations with 0.9 M LiBr, it was noted that, while the activity at pH 6.8 increased 500%, the activity measured at pH 8.2 was decreased by 20–50%. Preincubation with 1 M NaI or NaSCN caused nearly complete inhibition of catalytic activity.

Attempts to Dissociate Phosphorylase Kinase with Neutral Salts. Because treatment with trypsin activates phosphorylase kinase (Krebs et al., 1964) and tryptic fragments of kinase retain catalytic activity (Graves et al., 1973), we checked the possibility that salts could cause stimulation of activity by promoting dissociation of the kinase to a more active state. There has been a previous report of unsuccessful attempts to dissociate nonactivated kinase by use of high concentrations of neutral salts (Graves et al., 1973); however, the salts used in that study (NaCl, $NaClO_4$, and KI) are all inhibitory at high concentrations. In the present work, analytical ultracentrifugation was used to determine if dissociation could be induced by 1 M NH_4NO_3 or LiBr. Both these salts caused aggregation of the enzyme. A low concentration of $NaNO_3$ (0.1 M) did not change the protein's sedimentation properties.

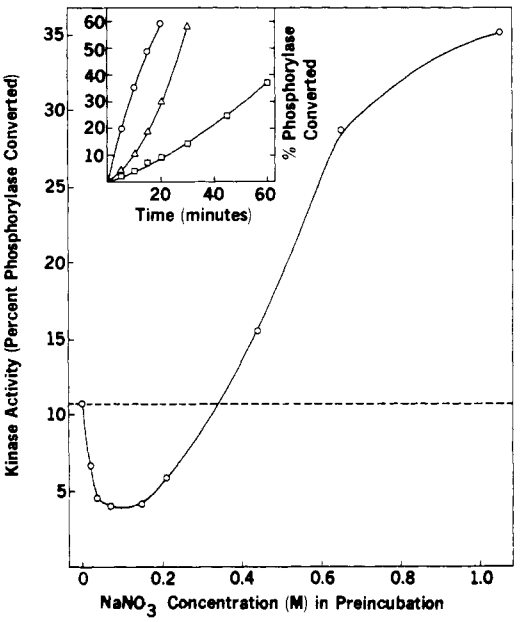

FIGURE 3: Effect of various $NaNO_3$ concentrations on the activity of nonactivated phosphorylase kinase at pH 6.8. Nonactivated kinase (27 μg/ml) was preincubated at 30 °C with $NaNO_3$ at the concentrations indicated. The preincubation buffer was 20 mM Hepes–15 mM mercaptoethanol, pH 6.8. After 30 min, the kinase was assayed by diluting threefold into a reaction mixture containing phosphorylase (14.9 mg/ml), buffer (50 mM Hepes, pH 6.8), mercaptoethanol (5 mM), and MgATP (6.7 mM $Mg(CH_3CO_2)_2$, 2.3 mM [^{32}P]ATP). The incorporation of ^{32}P was determined after 10-min assay time. The insert shows the time courses for the control reaction and for the reactions in which maximal stimulation and inhibition by $NaNO_3$ occurred. Symbols: (○) 1.05 M $NaNO_3$ in preincubation; (△) no $NaNO_3$; (□) 0.15 M $NaNO_3$ in preincubation.

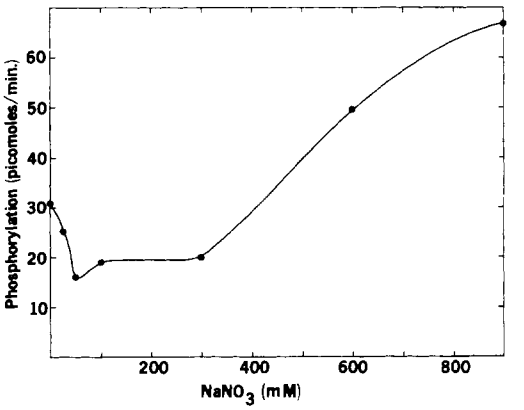

FIGURE 4: Effect of $NaNO_3$ on autophosphorylation. Nonactivated kinase (0.4 mg/ml) was incubated with buffer (20 mM Hepes, pH 6.8), mercaptoethanol (10 mM), MgATP (10 mM $Mg(CH_3CO_2)_2$, 3 mM [^{32}P]ATP), and various amounts of $NaNO_3$ as indicated. At various intervals, 25-μl aliquots were removed from this assay mixture for the determination of the rate of ^{32}P incorporation.

The influence of neutral salts on the behavior of activated phosphorylase kinase was also studied with gel filtration. Sephadex G-200 columns (1.1 × 30 cm) were equilibrated with pH 8.6 glycine (50 mM), mercaptoethanol (20 mM), and either KBr (3 M) or LiBr (1.5 M). When ^{32}P-labeled phosphorylated kinase (80 μg) was passed through these columns, all the radioactivity eluted in a single, sharp peak coinciding with the void volume. So, no evidence of dissociation by neutral salts was obtained.

Neutral Salts and Autophosphorylation. Besides being phosphorylated by protein kinase, phosphorylase kinase is able to phosphorylate itself (Walsh et al., 1971). Recent work from

this laboratory has suggested that the lag observed in the time course of the phosphorylase kinase reaction is due to autophosphorylation (Carlson et al., 1975; Carlson and Graves, 1976). Because neutral salts influence this lag (Figure 3, insert), we decided to study the effect of neutral salts on autophosphorylation. Once again, a biphasic response was observed as the concentration of $NaNO_3$ was increased. The rate of autophosphorylation at pH 6.8 decreased as the concentration of $NaNO_3$ was raised from 0 to 0.5 M (Figure 4). The inhibition grew progressively less as the $NaNO_3$ concentration was increased to approximately 0.4 M. At concentrations greater than this, the rate of autophosphorylation was stimulated above the control value obtained in the absence of $NaNO_3$. The maximal stimulation in this experiment, which occurred at 0.9 M $NaNO_3$, was twofold. LiBr proved more effective than $NaNO_3$ in causing this stimulation. At 0.9 M LiBr, a fourfold increase in the rate of autophosphorylation was observed. So, not only do high concentrations of neutral salts stimulate the rate of phosphorylase conversion by phosphorylase kinase, but they also stimulate the rate of phosphorylase kinase autophosphorylation.

Discussion

The disruption of protein structure and inhibition of enzymic activity by neutral salts is a widely observed phenomenon. The order of increasing effectiveness of anions in bringing about these changes in a diverse sampling of macromolecules is described by the anionic Hofmeister series. The inhibition of nonactivated phosphorylase kinase activity at pH 7.5 by 0.1 M neutral salts (Table I) follows the order in which the salts influence other macromolecules (Warren and Cheatum, 1966; von Hippel and Schleich, 1969). The only major displacement on the anionic list for inhibition of nonactivated kinase is SO_4^{2-}; however, SO_4^{2-} could be influencing the substrate because it is known to stimulate phosphorylase activity (Engers and Madsen, 1968).

It was concluded, because of the following reasons, that the locus of sensitivity to low concentrations of anions resides in the phosphorylase kinase molecule and not in its substrate, phosphorylase. (1) Nonactivated kinase was more sensitive to salt inhibition than the activated form. (2) Ca^{2+} partially overcame the inhibition of nonactivated kinase. (3) Inhibition by Cl^- occurred with either phosphorylase or the tetradecapeptide as substrate. (4) Phosphorylation of nonactivated phosphorylase kinase by protein kinase was markedly inhibited by $NaNO_3$, but this salt had little effect on the phosphorylation of histone by protein kinase.

Nonphosphorylated kinase was much more sensitive to salt inhibition than was its phosphorylated counterpart. Consequently, the anions probably inhibit enzymic activity by disrupting a portion of the enzyme's structure, rather than by interacting directly with the catalytic site. If the anions exerted their effect by interacting with the catalytic site, then one would have expected the two forms of kinase to be inhibited equally. The finding of differential salt effects on two forms of an enzyme has previously been used by Warren et al. (1966) to argue that the anionic inhibition of myosin ATPase is through structure disruption. They found that native myosin was more sensitive to inhibition by salts than was a chemically modified form of the enzyme.

A recent report by Jennissen and Heilmeyer (1975) provides further evidence of influence by the anionic Hofmeister series on phosphorylase kinase. They noted that the elution, with salts, of nonactivated kinase from a hydrophobic affinity column followed the order of the anionic Hofmeister series.

Effectors of enzymic activity are, without exception, also effectors of autophosphorylation, and in the same direction. Low concentrations of neutral salts, EGTA (Meyer et al., 1964; Walsh et al., 1971), and low pH (Krebs et al., 1959; Carlson and Graves, 1976) inhibit kinase activity and autophosphorylation. Glycogen (Krebs et al., 1964; DeLange et al., 1968), Ca^{2+} (Ozawa et al., 1967; Krebs et al., 1968; Walsh et al., 1971), high pH (Krebs et al., 1959; Carlson and Graves, 1976), and several peptide substrates (Carlson et al., 1975) all stimulate activity and autophosphorylation. Recent work from this laboratory has suggested that the lag in the rate of product formation in the phosphorylase kinase reaction is due to autophosphorylation (Carlson et al., 1975; Carlson and Graves, 1976). The findings of the present work are consistent with that idea. Low concentrations of neutral salts inhibit autophosphorylation and cause the lag to be less pronounced. High concentrations of neutral salts stimulate autophosphorylation and abolish the lag.

The biphasic response of the enzyme to increasing concentrations of $NaNO_3$ (Figure 3) was unexpected. Although the greatest stimulation by $NaNO_3$ was 5-fold, this amounted to a 12-fold stimulation over the reaction that showed the largest inhibition by $NaNO_3$. We thought it remarkable that such a large stimulation of activity could have occurred because one would normally expect the inhibitory process to become more evident as the concentration of the effector was increased. Additional experiments must be performed to fully understand the mechanism through which high concentrations of neutral salts stimulate activity and autophosphorylation. It is possible that the salts cause a conformational change in the kinase molecule, which leaves it poised for subsequent autophosphorylation and resultant activation. In this case, the autoactivation would need be sufficiently rapid so that no lag would be evident in the activity assay. This might also be the situation that exists when the enzyme is assayed at high pH. In this instance, autophosphorylation has been shown to be rapid, and the conversion of phosphorylase is linear. On the other hand, one could argue that the increased rate of autophosphorylation after preincubation with high concentrations of salts is either merely coincidental or a natural consequence of increased enzymic activity. We do not favor this last explanation, however, because autophosphorylation and resultant activation of phosphorylase kinase can occur in the absence of any effectors. High concentrations of salts seem to cause the enzyme to aggregate and could, therefore, influence the rate of autophosphorylation in that manner. This would be especially likely if autophosphorylation were an intermolecular process.

Acknowledgments

The authors thank L. B. Tabatabai for generously providing time and help and for her continued interest in this problem. G. W. Tessmer is to be thanked for providing purified tetradecapeptide.

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A Detailed Structural Comparison between the Charge Relay System in Chymotrypsinogen and in α-Chymotrypsin†

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ABSTRACT: An improved 2.5-Å electron density map of chymotrypsinogen was calculated by incorporating heavy-atom anomalous scattering effects and a new model of the molecule was constructed. Phases from x-ray structure factors (R = 0.43) computed from this model were then used in the calculation of another electron density map against which the model was further refined. The catalytic Ser-195 side chain in the new model is in the "down" or "acyl" orientation and its Oγ atom is in position to form a normal hydrogen bond with Nϵ2 of His-57. In contrast, the corresponding hydrogen bond in α-chymotrypsin (Birktoft, J. J., and Blow, D. M. (1972), *J. Mol. Biol. 68,* 187) is severely distorted, probably as a consequence of a 1.5-Å shift in the relative positions of the two cylindrical folding domains composing most of the molecule. We suggest that this activation induced distortion of the charge-relay, hydrogen-bonding system plays an important role in the genesis of enzymic activity, in accord with an earlier proposal by Wang concerning the role of bent hydrogen bonds in enzyme catalysis (Wang, J. H. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 874).

Bovine chymotrypsinogen A[1] is the zymogen precursor of the pancreatic serine protease chymotrypsin A_π, and of the other A-type chymotrypsins, notably A_δ, A_α, and A_γ, in which further autocatalytic peptide cleavages have occurred. The key chemical event responsible for activation of the zymogen is a single tryptic cleavage at the peptide bond Arg-15–Ile-16.

In earlier publications from this laboratory (Freer et al., 1970; Kraut, 1971), the crystal structure of chymotrypsinogen at a nominal 2.5-Å resolution was described and compared with the 2.0-Å structure of tosylchymotrypsin (Sigler et al.,

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[1] The terms chymotrypsinogen or zymogen will hereinafter be used to designate bovine chymotrypsinogen A; bovine chymotrypsin A_α will be called chymotrypsin or simply "the enzyme".