Sequential Chemical Modifications of Tyrosyl Residues in Alkaline Phosphatase of *Escherichia coli**

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ABSTRACT: Six tyrosyl residues of alkaline phosphatase of *Escherichia coli* were nitrated with tetranitromethane, subsequently reduced to aminotyrosine with sodium hydrosulfite, and finally N acylated with acetic and succinic anhydrides. The derivates resulting from each of these sequential tyrosyl modifications are all both active phosphohydrolases and phosphotransferases. Compared with the native enzyme, the nitrotyrosyl enzyme exhibits 60 and 95%, the aminotyrosyl enzyme 130 and 350%, and the acylaminotyrosyl enzyme 65 and 110% hydrolase and transferase activity, respectively. The presence of substrates affects neither the time course, nor the enzymatic consequences of these chemical modifications nor do they alter the K_m of the enzyme, indicating that these tyrosyl residues are not involved directly either in catalytic activity or in substrate binding. Sedimentation analysis, gel filtration, and circular dichroism studies do not reveal structural differences between the aminotyrosyl and the native enzyme. The visible absorption spectra of native, aminotyrosyl-, iodo-, and *N*-bromosuccinimide-treated cobalt phosphatase resemble one another closely, suggesting that the tyrosyl modifications do not affect the metal binding site of the enzyme. Thus, the enhanced catalytic rates of aminotyrosyl-, iodo-, and *N*-bromosuccinimide-modified phosphatase may reflect modification of residues not located at the active center, but which perhaps exert their effects on a catalytic rate-limiting, conformational change.

Alkaline phosphatase of *Escherichia coli* is a zinc metalloenzyme which catalyzes both the hydrolysis of phosphate monoesters and the transfer of phosphate from such esters to acceptor alcohols (Wilson *et al.*, 1964). Recent observations have suggested the presence of two functionally distinct classes of metal ions in the enzyme: two of the zinc atoms appear to be required for catalytic activity while the other two, apparently present in variable amounts in different enzyme preparations, seem to stabilize quaternary structure (Simpson and Vallee, 1968; Simpson, 1969; Simpson and Vallee, 1969). Replacement of zinc by cobalt changes the specificity of the enzyme: cobalt phosphatase is an active phosphohydrolase but has practically no phosphotransferase activity (Tait and Vallee, 1966).

Aside from the presence of the metal atom, the information regarding the identity of the components of the active center of alkaline phosphatase is not abundant. Inorganic phosphate or phosphate esters phosphorylate a seryl residue of the enzyme, which is stable at pH ≤ 5.5 (Engström and Agren, 1958; Schwartz and Lipman, 1961; Pigretti and Milstein, 1965). Experimental evidence increasingly indicates that both the hydrolytic and transfer reactions share the phosphorylation of this specific seryl group as an integral step in the reaction pathway (Wilson *et al.*, 1964; Barrett *et al.*, 1969; Levine *et al.*, 1969; Reid *et al.*, 1969).

In the present study, six tyrosyl residues of alkaline phosphatase have been selectively nitrated with tetranitromethane. The resultant 3-nitrotyrosyl residues were subsequently reduced to the 3-amino derivatives (Sokolovsky *et al.*, 1967) and these, in turn, were modified by N acylation using conditions designed to achieve specificity (Sokolovsky *et al.*, 1967). Depending on the nature of the tyrosyl substitution, increased, decreased, or unaltered activity is observed. Based upon physical and kinetic evidence, the modified tyrosyl residues do not seem to be involved directly in catalysis, in substrate binding, or in interaction with the active site metal atoms. Rather, these chemical modifications appear to modulate activity indirectly, perhaps by affecting the macromolecular structure of the enzyme.

Materials

Alkaline phosphatase from *Escherichia coli*, strain C-90, was prepared according to Simpson *et al.* (1968). The enzyme obtained by this method contained 4 g-atoms of zinc/mole of enzyme. The specific activities of the preparations used ranged from 45 to 55 μmoles of 4-nitrophenyl phosphate cleaved per min per mg of protein when measured in 1 M Tris-Cl (pH 8.0) 25°, at a substrate concentration of 1 mM. Other materials and suppliers were: 4-nitrophenyl phosphate disodium tetrahydrate (Sigma Chemical Co.); adenosine 5′-triphosphate disodium salt, glucose 1-phosphate disodium salt, phosphoenolpyruvate tricyclohexylammonium salt (Boehringer); tetranitromethane, *p*-dimethylaminobenzaldehyde (Aldrich Chemical Co.); *N*-bromosuccinimide, acetic anhydride, succinic anhydride, 8-hydroxyquinoline-5-sulfonic acid, phenylphosphonic acid (Eastman Organic Chemicals); iodine, sodium hydrosulfite (Fisher Scientific Co.); reduced glutathione (Schwartz BioResearch, Inc.); Chelex-100, 100–200 mesh, sodium form (Bio-Rad Laboratories); cobalt(II) chloride was prepared by dissolving spectrographically standardized cobalt sponge (Johnson and Matthey Co.) in 6 N HCl. After removal of the HCl in a rotary evaporator, the $CoCl_2$ was dissolved in metal-free water. The monocyclohexylammonium salt of 2,4-dinitrophenyl phosphate prepared

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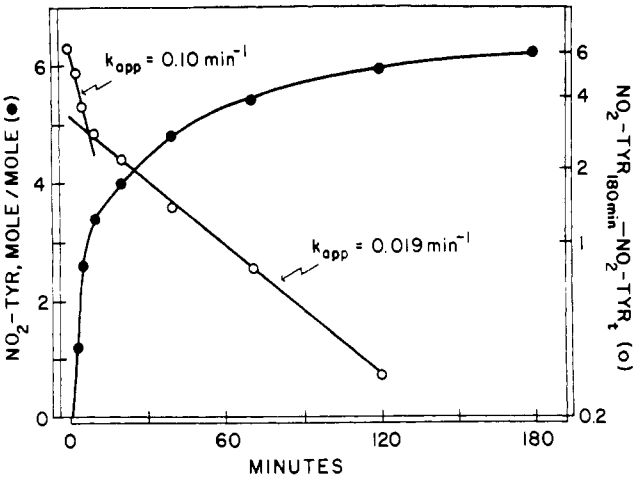

FIGURE 1: Time course of nitration of alkaline phosphatase by TNM (●). A 100-fold molar excess of TNM (100 μl of a 0.168 M solution in 95% ethanol) was added to 6.0 ml of a phosphatase solution, 2.5 mg/ml, 2.8 × 10^{-5} M, in 0.05 M Tris-Cl–1 M NaCl (pH 9.0). The reaction mixture was kept at 20°, and at the times indicated aliquots were removed, treated with reduced glutathione (final concentration 0.03 M) to consume residual TNM, diluted threefold, and dialyzed against 0.1 M Tris-Cl–1 M NaCl (pH 8.0). 3-Nitrotyrosyl content was determined spectrophotometrically. Two rate constants, K_{app}, of 0.10 and 0.019 min^{-1} are evident (○).

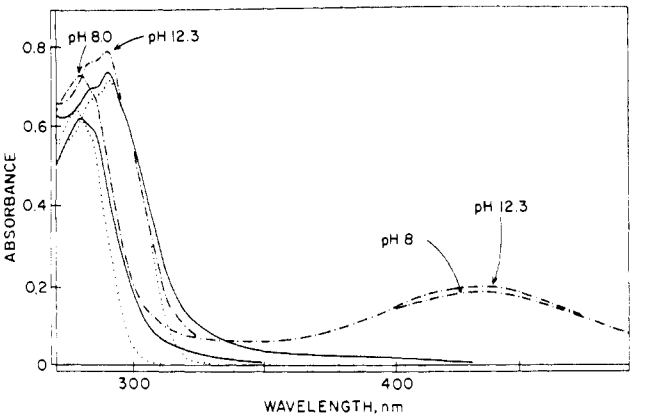

FIGURE 2: Absorption spectra of native (···), nitrotyrosyl (·—·—·), and aminotyrosyl (—) phosphatase at pH 8.0 and 12.3. The enzyme derivatives contain 5.6 3-nitrotyrosyl or 3-aminotyrosyl residues, respectively. Protein concentration was 8.5 × 10^{-6} M in 0.05 M Tris-Cl–1 M NaCl (pH 8.0); NaOH (19 M) was added to give a pH of 12.3.

according to Bunton *et al.* (1967) was a gift from Dr. Michael Gottesman.

Methods

Enzymatic activity was measured with the substrate 4-nitrophenyl phosphate in a Unicam SP 800 spectrophotometer at 25°. The release of 4-nitrophenol was determined using $\epsilon_{400}^{pH\ 8.0}$ value of 1.68 × 10^4. The assay mixture contained 1 mM substrate in 0.01 M Tris-Cl (pH 8.0) for determination of substrate consumption by simultaneous ester hydrolysis and phosphate transfer. The transferase activities reported here represent the differences between the rates of 4-nitrophenol release in 1 M Tris and in 0.01 M Tris. The values found by this method are 70–80% of those found by measuring directly the difference between the amounts of 4-nitrophenol and P_i produced in the 1 M Tris assay.

Protein concentrations were measured spectrophotometrically using $A_{278}^{1\%} = 7.2$ (Plocke *et al.*, 1962) or by the method of Lowry *et al.* (1951). The molecular weight of the enzyme was taken to be 89,000 (Simpson, 1969).

Apophosphatase was prepared by dialysis of the native protein against 0.01 M sodium 8-hydroxyquinoline-5-sulfonate (pH 8.0) at 25°, followed by exhaustive dialysis against metal-free water to remove the chelating agent (Simpson and Vallee, 1968). Before this procedure protein solutions were concentrated to a concentration of 10–20 mg/ml using a Diaflow ultrafiltration apparatus (Amicon Corp.). Glassware and buffers were rendered metal free as reported previously (Thiers, 1957; Himmelhoch *et al.*, 1966).

Cobalt alkaline phosphatase was prepared by dialysis of the apoenzyme against a 100-fold volume of 0.01 M Tris-Cl–1 mM $CoCl_2$ (pH 8.0) for 6 hr, followed by two 3-hr dialyses against 0.01 M Tris-Cl (pH 8.0). For determination of the activity of cobalt phosphatase in 1 M Tris, 10^{-5} M $CoCl_2$ was added to the assay to compensate for the complex formation of cobalt with Tris as previously described (Gottesman *et al.*, 1969).

Nitrotyrosyl content was determined spectrophotometrically after dialysis against 0.05 M Tris-Cl–1 M NaCl (pH 8.0) using $\epsilon_{428}^{pH\ 8.0} = 3800$ (Riordan *et al.*, 1966).

Amino acid analyses were performed using a Spinco Model 120B amino acid analyzer, after hydrolysis in sealed, evacuated tubes with 6 N HCl *in vacuo* at 110° for 22 hr. The degree of modification of free amino groups was determined by means of the ninhydrin reaction (Moore and Stein, 1948) using phenylalanine as a standard.

Measurements of pH were made with a Radiometer Model 22 pH meter. Absorbance at discrete wavelengths was measured with a Zeiss PMQ II spectrophotometer. Absorption spectra were obtained with a Cary Model 14R spectrophotometer. Spectrophotometric titrations were performed using a small volume titration cell described by Auld and French (1970). Circular dichroism measurements were performed with a Cary Model 60 spectropolarimeter equipped with a Cary Model 6001 circular dichroism accessory.

Sedimentation velocity was determined with a Spinco Model E ultracentrifuge. The distribution coefficients on gel filtration were determined with a Sephadex G-100 column (2 × 50 cm) at room temperature with the hydrostatic pressure adjusted to a flow rate of 30 ml/hr (Andrews, 1964). The void volume was measured using Blue Dextran 2000 (Pharmacia); the elution volume, V_e, was determined by assaying the eluate fractions in runs with high enzyme concentration (7–9 mg/ml) for protein concentration and with low enzyme concentration (0.023–0.025 mg/ml) for enzymatic activity.

Results

Nitrotyrosyl Phosphatase. E. coli alkaline phosphatase contains 20 tyrosyl residues/mol wt 89,000 (Simpson *et al.*, 1968). Exposure of the enzyme to a 100-fold molar excess of tetranitromethane at pH 9.0 results in the nitration of about six tyrosyl residues after 3 hr. The time course of the reaction indicates an initial rapid nitration of two residues with an apparent rate constant of 0.10 min^{-1}, followed by nitration of four more residues with an apparent rate constant of 0.019 min^{-1}, fivefold slower than that for the first two residues.[1]

[1] Under the same conditions and based on nitroformate production, the pseudo-first-order rate of reaction of TNM with tyrosyl model compounds such as *N*-acetyltyrosine, *N*-acetyltyrosinamide, and *N*-acetyltyrosine ethyl ester is 0.27 min^{-1}.

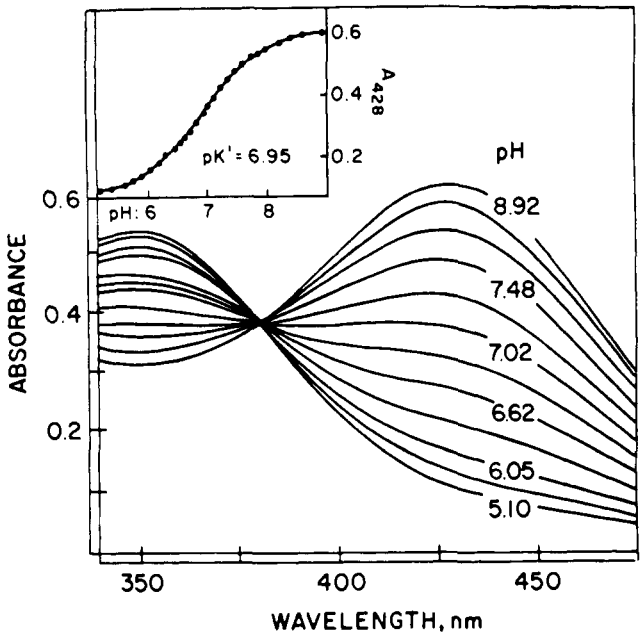

FIGURE 3: Spectral titration of nitrotyrosyl phosphatase. Nitrotyrosyl phosphatase, containing 6.0 nitrotyrosyl residues/molecule, was titrated in 0.001 M Tris-Cl–1 M NaCl with 0.25 N NaOH and 0.25 N acetic acid at the pH values indicated. Insert: the points represent the values determined experimentally, the solid line a theoretical titration curve of a group with a pK of 6.95.

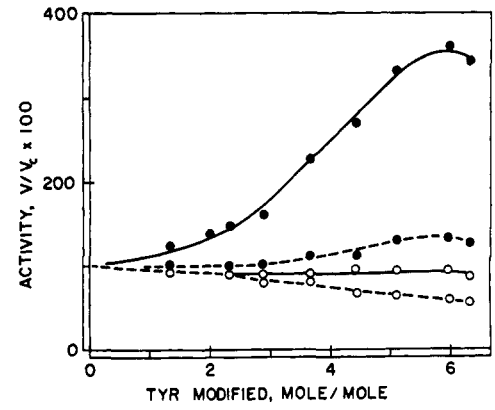

FIGURE 4: Dependence of hydrolase (– – –) and transferase (——) activity on number of nitrotyrosyl (○) or aminotyrosyl (●) residues. Nitrotyrosyl phosphatase nitrated to varying degrees was prepared as in Figure 1. The addition of sodium hydrosulfite (final concentration 0.01 M) resulted in instantaneous decolorization and activity increase which remained unaltered by subsequent dialysis.

Under the conditions employed, the remaining fourteen tyrosyl residues of the enzyme are not nitrated. The rate of nitration increases with pH. At pH 8, 9, and 10, modification of six tyrosyl residues per mole of enzyme requires 16 hr, 3 hr, and 3 min, respectively. For the present studies, nitration for 3 hr at pH 9.0 (Figure 1) was adopted as the standard procedure, yielding a derivative with 5.5–6.0 nitrotyrosyl residues/molecule, subsequently referred to as *nitrotyrosyl phosphatase*.

The visible absorption spectrum of nitrotyrosyl phosphatase at pH 12 exhibits a maximum at 428 nm, characteristic of the nitrotyrosyl chromophore (Figure 2). Acidification shifts the absorption maximum to 348 nm, with an isosbestic point at 381 nm (Figure 3). A plot of A_{428} *vs.* pH is in good agreement with a theoretical titration curve for a group with an apparent pK of 6.95 (Figure 3, insert) and suggests a uniform pK for all the nitrotyrosyl residues, slightly lower than the pK of 7.2 reported for the phenolic hydroxyl group of the free amino acid, 3-nitrotyrosine (Sokolovsky *et al.*, 1967).

Nitration of the first two tyrosyl residues has little effect on hydrolase activity, but nitration of the next four tyrosines decreases hydrolase activity to 60% of the control (Figure 4). In contrast, phosphotransferase is not altered significantly throughout (Figure 4).

Aminotyrosyl Phosphatase. Reduction of nitrotyrosyl phosphatase with sodium hydrosulfite (Sokolovsky *et al.*, 1967) drastically alters both its spectral and its enzymatic properties. The visible absorption spectrum of nitrotyrosyl phosphatase (Figure 2) is abolished. Transferase activity increases about threefold, and hydrolase activity is restored to approximately the same level as that of the native enzyme (Figure 4). The new derivative, containing 5.5 to 6.0 aminotyrosyl residues, will be referred to as *aminotyrosyl phosphatase*. Reduction of the first two residues nitrated affects activity but little. Thereafter, when derivatives containing more than two nitrotyrosyl residues are reduced, activity increases as a function of the number of aminotyrosyl residues per enzyme molecule formed (Figure 4), becoming maximal (3.5-fold) on reduction of the enzyme containing six nitrotyrosyl residues.[2]

Sodium hydrosulfite is also known to reduce disulfide bonds in proteins (Windus and Turley, 1941; Harris and Brown, 1947). However, under the present conditions none of the four disulfide bonds of phosphatase (Schlesinger and Barrett, 1965) appear to be reduced. No mercaptide formation is spectrophotometrically detectable (Boyer, 1954) after reduction of nitrotyrosyl) phosphatase in the presence of 5 mM *p*-mercuribenzoate, a 500-fold molar excess with respect to enzyme. The activity changes are identical whether nitrotyrosyl phosphatase is reduced in the presence or absence of *p*-mercuribenzoate.

Amino acid analysis of nitrotyrosyl and aminotyrosyl phosphatase reveals that the loss of tyrosine corresponds closely to the nitrotyrosyl content determined spectrophotometrically (Table I). However, the recovery of nitrotyrosine was only about 70% of that expected from spectrophotometric analysis.

While many proteins studied thus far react readily with low molar excesses of tetranitromethane at pH 8 (Sokolovsky *et al.*, 1966), the reactivity of the tyrosyl residues of alkaline phosphatase is relatively low, necessitating nitration with a 100-fold molar excess of tetranitromethane at pH 9 (Figure 1). Under these conditions, as might be expected (Sokolovsky *et al.*, 1970), 1.1–1.4 tryptophyl residues/molecule are lost consistently.[3] Nitration with a 16-fold molar excess of tetranitromethane at five times higher protein concentration, 13.0 mg/ml, 1.56×10^{-4} M, results in the loss of only 0.6 mole of

[2] The activity of aminotyrosyl phosphatase, containing about six 3-aminotyrosyl residues, varied between 250 and 350% of that of the native enzyme, depending on the particular preparation of enzyme used.

[3] Tryptophan modification under these conditions is consistent with the increased rate of the reaction between tryptophan and high concentrations of TNM at pH >8 (Sokolovsky *et al.*, 1970). However, tryptophanyl modification does not seem to be responsible for the observed activity changes since the degrees of modification and activity change do not correlate. The replacement of tryptophan by azotryptophan or tryptazan in the biosynthesis of alkaline phosphatase from *E. coli* supports this conclusion, since the resultant enzyme does not differ significantly either in structure or function from the native, tryptophan-containing enzyme (Schlesinger, 1968).

TABLE I: Comparative Amino Acid Analysis of Native, Nitrotyrosyl, and Aminotyrosyl Phosphatase.[a]

	Native[c]	Nitro-tyrosyl	Amino-tyrosyl
Lys	49.4	52.0	51.1
His	16.4	16.3	16.4
Arg	25.0	22.5	23.2
Asp	94.7	92.0	94.4
Thr	92.2	90.4	91.0
Ser	34.6	37.4	36.9
Glu	91.0	86.9	89.4
Pro	38.0	42.3	40.7
Gly	85.7	87.5	86.1
Ala	124.4	123.0	120.7
Cys/2	6.8	6.6	6.6
Val	42.5	45.4	42.3
Met	14.2	14.8	14.3
Ile	26.3	26.0	27.0
Leu	74.2	75.8	75.5
Phe	16.0	16.9	16.4
Trp[b]	7.2	5.8	Not det
Tyr	20.0	14.3	13.7
NO_2-Tyr	0	4.2	0
ΔTyr (native nitro)		5.7	
ΔTyr (native amino)			6.3
NO_2Tyr (A_{428})	0	6.2	0

[a] Nitrotyrosyl and aminotyrosyl phosphatase were prepared as in the legends to Figures 1 and 4. Before hydrolysis (see Methods) the samples were dialyzed against water. The calculations are based on a molecular weight of 89,000 (Simpson and Vallee, 1968; Simpson and Bethune, 1970). [b] Colorimetric determination with dimethylaminobenzaldehyde (Spies and Chambers, 1949). [c] The results are nearly identical with those of Rothman and Byrne (1963) and Simpson *et al.* (1968).

TABLE II: Activities of Zinc and Cobalt Phosphatase Derivatives.

Phosphatase Derivative	Specific Activity (U/mg)			
	Zinc Enzyme		Cobalt Enzyme[d]	
	Hydro-lase	Trans-ferase	Hydrolase	Trans-ferase
Native	25	25	6.7	0.7
Nitrotyrosyl[a]	13	18	4.7	0.3
Aminotyrosyl[a]	22	61	10.5	3.8
N-Acetylamino-tyrosyl[b]	13	18	3.7	0.4
N-Succinylamino-tyrosyl[c]	13	26		

[a] Nitrotyrosyl and aminotyrosyl derivatives of the zinc enzyme were prepared as in Figures 1 and 4. [b] Aminotyrosyl enzyme, 10^{-5} M in 0.05 M Tris–1 M NaCl (pH 8.0), was mixed with an equal volume of saturated sodium acetate (pH 6.0). After cooling to 0°, 1 μl of acetic anhydride per ml was added. After 30 min at 0° activities were determined. Controls showed no loss of activity. [c] Succinic anhydride (3 mg/2 ml) was added to a 10^{-5} M solution of aminotyrosyl enzyme in 0.2 M sodium acetate (pH 5.0). Activities were determined after stirring for 30 min at 0°. Controls showed no loss of activity. Acetylation and succinylation of native and nitrotyrosyl enzyme did not alter their activities. [d] Cobalt enzyme derivatives were prepared as indicated under Methods.

tryptophan/mole of enzyme, though 5.5 tyrosyl residues are still nitrated and the enzymatic consequences of the modification are the same. Residues other than tyrosyl and tryptophanyl do not appear to be modified.

N-Acylaminotyrosyl Phosphatases. The tyrosyl residues involved in modulation of activity by virtue of nitration and subsequent reduction can be subjected to a third sequential modification. Acylation of aminotyrosyl phosphatase with acetic or succinic anhydride at pH 6 or 5, respectively, returns the increased transferase activity to the level of nitrotyrosyl phosphatase (Table II).

Hydroxylamine (0.5 M at pH 8.0, 20°, 2 hr) does not reverse these effects on activity, suggesting that they are the result of N acylation. The number of free ninhydrin-positive amino groups (Moore and Stein, 1948) decreases from 28 phenylalanine equiv in native and 29 in aminotyrosyl phosphatase to 22 in succinylaminotyrosyl phosphatase, indicating some acylation of ε- and α-amino groups in addition to that of the aromatic amino groups. However, such acylation does not significantly affect the activities of native and nitrotyrosyl phosphatase (Table II) suggesting that the decrease in the activity of the aminotyrosyl enzyme is due specifically to N acylation of the aminotyrosyl residues.

Tyrosyl Modifications of Cobalt Alkaline Phosphatase. A combination of inorganic and organic modifications have served further to examine possible effects of tyrosyl modification on the metal ions of phosphatase. Replacement of zinc of native phosphatase with cobalt abolishes transferase but not hydrolase activity (Tait and Vallee, 1966; Simpson and Vallee, 1968). Nitration of cobalt phosphatase decreases hydrolase activity, but subsequent reduction restores it and at the same time generates transferase activity. Conversion of the aminotyrosyl enzyme to *N*-acetylaminotyrosyl cobalt phosphatase reduces both activities to those of the nitrotyrosyl cobalt enzyme (Table II). Addition of cobalt to apoaminotyrosyl, apoiodo-, or apo-*N*-bromosuccinimide phosphatase generates a visible absorption spectrum similar to that of native cobalt phosphatase. Addition of 2 moles of phosphate to these cobalt enzyme derivatives changes the positions and absorptivities of the absorption bands in identical fashion.

Physicochemical Properties of Nitrotyrosyl and Aminotyrosyl Phosphatase. The circular dichroic spectra of native, nitrotyrosyl and aminotyrosyl phosphatase are very similar in the region from 215 to 240 nm (Figure 5). However, each has a distinct and characteristic spectrum in the region from 260 to 290 nm. Moreover, the nitrotyrosyl enzyme displays a broad weak, negative Cotton effect centered around 430 nm which is absent in the native enzyme or the aminotyrosyl derivative (Figure 5). Phosphate ion, 0.01 M, does not alter the circular dichroic spectra of any of these enzymes.

The sedimentation velocities of native, nitrotyrosyl and aminotyrosyl phosphatases, determined at protein concentrations from 7 to 9 mg per ml, do not differ significantly (Table III). Further, on gel filtration, their distribution coefficients

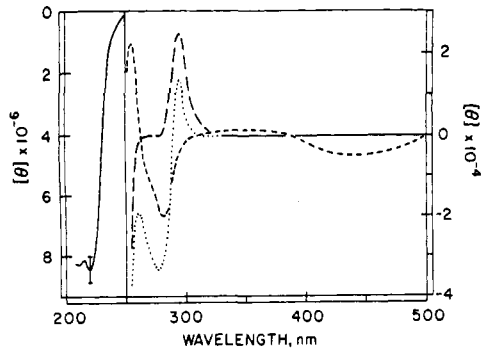

FIGURE 5: Circular dichroic spectra of native (···), nitrotyrosyl (– – –), and aminotyrosyl (——) phosphatase. The solid line indicates complete overlap of the curves. Enzyme concentration is 10^{-4} M, in 0.05 M Tris-Cl–1 M NaCl (pH 8.0). The following cells were used; 600–300 nm, 1 cm; 330–240 nm, 0.2 cm; 250–210 nm, 0.02 cm. The Cotton effect of nitrotyrosyl phosphatase at 430 nm was confirmed at half the protein concentration.

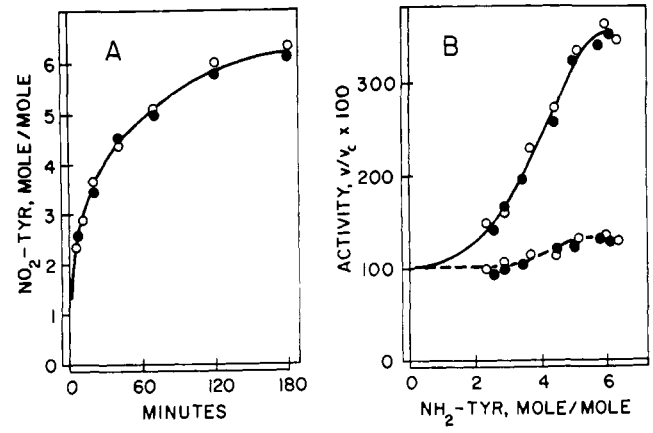

FIGURE 6: Nitration in the presence (●) and absence (○) of phosphate, 0.01 M. Conditions as in Figure 1. (A) Time course of nitration. (B) Hydrolase (– – –) and transferase (——) activity after reduction of the enzyme samples of Figure 6A. The number of aminotyrosyl residues per molecule was taken as the number of nitrotyrosyl residues per molecule before reduction.

also agree closely with one another when determined at enzyme concentrations close to those employed for the assay of enzymatic activity (Table III). By these criteria, the present tyrosyl modifications do not alter the quaternary structure of the enzyme.

Substrate Binding and Tyrosyl Modification. The presence of 0.01 M phosphate (or 0.025 M phenylphosphonate) affects neither the time course of nitration (Figure 6A) nor the relation of the resultant activity increase to the number of aminotyrosyl residues per enzyme molecule, found subsequent to reduction (Figure 6B). Similarly, as judged by the production of nitroformate in the reaction mixture (Sokolovsky *et al.*, 1966), the substrates glucose 6-phosphate, 0.1 M, pyrophosphate, 0.4 M, or 0.01 M phosphate–1 M Tris-Cl do not affect the time course of nitration. Further, the K_m values for 4-nitrophenylphosphate of native, nitrotyrosyl and aminotyrosyl phosphatase do not vary significantly when determined at pH 8.0, either in 0.01 M Tris-Cl–1 M NaCl or in 1 M Tris-Cl (Table IV). Similarly, the inhibition constant, K_i, for phosphate ion is virtually unchanged by either of the two chemical modifications. For both the native enzyme and the derivatives acceptor concentration affects the rate of 4-nitrophenol release in similar fashion (Figure 7); the maximal effect is observed at a Tris concentration of 1.5 M.

Hydrolase Activity of Aminotyrosyl Phosphatase at Low Ionic Strength. The rate-enhancing effect of tyrosyl amination does not appear to be limited to transferase activity. At low ionic strength hydrolase activity of the aminotyrosyl enzyme is also significantly higher than that of native phosphatase (Table V). The hydrolase activity of aminotyrosyl phosphatase toward all phosphate ester substrates examined is remarkably similar (Table V), as found for the native enzyme (Heppel *et al.*, 1962).

Discussion

The active center of alkaline phosphatase has been shown to contain a specific seryl residue which can be phosphorylated at acidic pH values (Engström and Agren, 1958; Schwartz and Lipman, 1961; Pigretti and Milstein, 1965); however, the identification of additional functional residues of this enzyme by means of chemical modification has proven problematic. Although 5-diazonium-1*H*-tetrazole, acetic anhydride, acetylimidazole, iodoacetate, and photooxidation in the presence of rose bengal have proven effective in modifying tyrosyl, lysyl, histidyl, and cysteinyl residues of many other proteins

TABLE III: Sedimentation Coefficients and Distribution Coefficients on Gel Filtration of Native, Nitrotyrosyl, and Aminotyrosyl Phosphatases.

Conditions	Phosphatase			Concn (mg/ml)
	Native	Nitrotyrosyl	Aminotyrosyl	
s^{20}_{app} 0.05 M Tris-Cl–1 M NaCl (pH 8.0)	5.7 S	6.0 S	5.8 S	7–9
K_d [a] 0.05 M Tris-Cl–1 M NaCl (pH 8.0)	0.18			7
0.05 M Tris-Cl–1 M NaCl (pH 8.0)	0.16	0.16	0.07	0.023–0.025
1 M Tris-Cl (pH 8.0)	0.14	0.15	0.16	0.023–0.025

[a] $K_d = (V_e - V_0)/(V_t - V_0)$. According to information supplied by the manufacturer of Sephadex, a K_d of 0.16 corresponds to a molecular weight of approximately 80,000. For phosphatase monomers (mol wt 45,000) a value of K_d = 0.26 would be expected.

TABLE IV: Michaelis Constants of Phosphatase Derivatives for 4-Nitrophenyl Phosphate.[a]

Phosphatase	K_m in 1 M NaCl (M × 10^5)	K_m in 1 M Tris-Cl (M × 10^5)
Native	1.0	2.0
Nitrotyrosyl[b]	0.7	1.5
Aminotyrosyl[b]	0.5	2.4

[a] Activity was measured at pH 8.0 by release of 4-nitrophenol (see Methods). [b] Containing 5.5 nitrotyrosyl or aminotyrosyl residues per molecule.

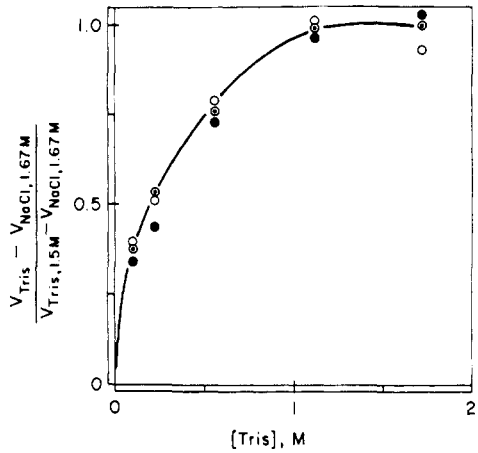

FIGURE 7: Activity of native (○), nitrotyrosyl (⊙), and aminotyrosyl phosphatase (●) as a function of acceptor concentration. The phosphatase derivatives are the same as in Table II. Activities were measured at the indicated Tris concentrations (pH 8.0), as detailed under Methods, the sum of Tris and NaCl concentrations being held constant at 1.67 M. Maximal increase of activity was observed when 1.67 M NaCl was replaced by 1.5 M Tris-Cl–0.17 M NaCl. The activity increments (maximally 93, 100, and 190% for native, nitrotyrosyl and aminotyrosyl enzyme, respectively) are normalized.

(Vallee and Riordan, 1969), none of these have materially altered the activity of alkaline phosphatase when used under mild conditions (Plotch and Lukton, 1965; Tait and Vallee, 1966; G. H. Tait and B. L. Vallee, unpublished observations).

Alone among the "tyrosyl" reagents, tetranitromethane has allowed examination of the role of these residues in catalytic function of alkaline phosphatase. Nitration of alkaline phosphatase results in a typical nitrotyrosyl absorption spectrum with a maximum at 428 nm (Figures 2 and 3). Spectral analysis and differential amino acid analysis coincide closely and appear to provide a reliable means for determining the nitrotyrosyl content of the protein. Nitration of alkaline phosphatase proceeds in two stages. Out of the total of 20 tyrosyl residues (Table I) two react readily, though at one third of the rate characteristic of tyrosine model compounds at pH 9.0. Four additional residues are nitratable but react even five times more slowly than the first two (Figure 1). The functional response to modification also indicates two classes of nitratable tyrosyl residues in alkaline phosphatase. Only the four slowly reacting residues appear to be involved in the activity changes observed on nitration and subsequent reduction (Figure 4).

Reduction of the nitrotyrosyl to aminotyrosyl residues with sodium hydrosulfite is the second of these sequential tyrosyl modifications. Sodium hydrosulfite reduces the nitrotyrosyl residues and does not appear to affect other groups (Table I). The disappearance of the absorption at 428 nm (Figure 2) serves to monitor the conversion of nitrotyrosyl to aminotyrosyl residues.

Among the functional consequences of chemical modification, the selective, 3-fold increase in transferase activity and 1.5- to 2-fold increase in hydrolase activity on reduction of the nitrotyrosyl to the aminotyrosyl enzyme stands out. These functional results are similar to those observed on treatment of phosphatase with *N*-bromosuccinimide or with iodine where the phosphatase activity is increased slightly and transferase activity is doubled (Tait and Vallee, 1966).[4]

Since all four of the residues whose modification (nitration and reduction) correlates with increases of activity apparently react at the same rate, the minimal number of aminotyrosyl residues which would be necessary to observe the increase in activity cannot be deduced from the present data. Similarly, spectral titration of nitrotyrosyl phosphatase fails to distinguish between any of the six nitrotyrosyl residues. Circular dichroism and hydrodynamic data do not indicate structural changes, though subtle alterations might well be beyond the level of resolution of these methods.

The physicochemical properties of 3-aminotyrosine differ from those of any other amino acid found in proteins. The pK of the aromatic amino group is 4.75 (Sokolovsky *et al.*, 1967), 3–5 pH units lower than the pK of aliphatic amino groups of protein residues. It is also distinctly lower than the pK values reported for the imidazole groups. This low pK allows preferential N acylation of the 3-aminotyrosyl residues with carboxylic acid anhydrides at low pH. During this third stage of sequential tyrosyl modifications, mainly the aromatic amino groups appear to be acylated. N acylation abolishes the effect of both nitration and reduction of these tyrosyl groups on activity (Table II), while leading to acylation of only a small fraction of the nonaromatic amino groups, as shown by the ninhydrin reaction. At lower pH values, no doubt the acylation of the aromatic amino group could be made more specific, thereby further lowering the reactivity of the α- and ϵ-amino groups. Unfortunately, the dissociation of metal ions at low pH precludes the choice of these conditions.

However, the zinc atom at the active site of the enzyme allows for yet other types of modification. While cobalt alkaline phosphatase is an active hydrolase, it is not a transferase (Tait and Vallee, 1966), though it exhibits a visible absorption spectrum serving as a probe for the active site and of its environment (Simpson and Vallee, 1968). Compared to the native cobalt enzyme, cobalt aminotyrosyl phosphatase has even greater hydrolase activity and, in contrast, also exhibits transferase activity (Table II). Quite similar enzymatic characteristics are observed with cobalt phosphatase when exposed to either iodine or *N*-bromosuccinimide (Tait and Vallee, 1966). The absorption spectra of aminotyrosyl-, iodo-, and *N*-bromosuccinimide-treated cobalt phosphatase closely resemble that of native cobalt phosphatase, as does their response to phosphate. In all instances 2 moles of phosphate

TABLE V: Native and Aminotyrosyl Phosphatase. Hydrolase Activities toward Various Phosphate Esters.[a]

	Sp Act. (U/mg)	
	Native	Aminotyrosyl[b]
4-Nitrophenyl phosphate	15.3	30.1
2,4-Dinitrophenyl phosphate	14.7	27.0
Glucose 1-phosphate	13.6	26.0
Phosphoenol pyruvate	14.0	24.6
ATP	12.1	20.1

[a] Activities were measured at substrate concentrations of 1 mM in 0.02 M Tris-Cl (pH 9.0) by determination of P_i released (Lowry and Lopez, 1946). [b] Prepared as described in the legend of Figure 4.

[4] Double-modification experiments imply that tetranitromethane and iodine modify the same tyrosyl residues.

induce a maximal and identical shift in band position. Apparently, the various modifications which increase activity do not detectably alter the metal–enzyme interaction.

The chemical modifications performed implicate tyrosyl residues in the functional consequences. However, a number of criteria suggest that the modified tyrosyl residues do not appear to be directly involved either in substrate binding or catalysis. The course of nitration is not influenced by the presence of inorganic phosphate or various phosphate esters (Figure 6), and the consequences of nitration in the presence and absence of phosphate do not seem to differ, as judged by the correlation between degree of tyrosyl amination and effect on activity (Figure 6). The presence of phosphate does not influence the circular dichroic spectra of native and modified phosphatase (Figure 5). Further, the Michaelis constants of the various phosphatase derivatives do not differ significantly from that of the native enzyme (Table IV). The relationship between the rate of 4-nitrophenyl phosphate cleavage and acceptor (Tris) concentrations also appears to be unaltered (Figure 7). It would appear that increased affinity of the phosphoryl intermediate for the acceptor alcohol does not seem to be the cause of the increased transferase activity of aminotyrosyl phosphatase.

The experimentally tangible effects of the various tyrosyl modifications appear to be limited to changes in the enzymatic activity. Tyrosyl modification does not abolish activity in any instance. While the pK' of the phenolic hydroxyl group is lowered from near 10.0 to about 7.0 as a consequence of nitration, it is raised back to about 10.0 when the nitro group is reduced to an amino group. One of the mechanisms by which nitration can affect the biological activity of a protein is by altering the pK' of an essential tyrosyl residue. In such instances, because of its effect on the phenolic hydroxyl pK', reduction might reverse the activity changes due to nitration either fully or partially. Thus, in the present case, nitration reduces hydrolase activity but leaves transferase activity unchanged. On reduction of the nitro- to the aminotyrosyl enzyme, hydrolase activity is restored to that characteristic of the native enzyme and transferase activity increases threefold. It is possible that alterations in pK' could account for these activity changes. The results of the current studies do not meet the criteria generally employed to localize a modified residue at the active site of any enzyme. Rather, the data suggest that the modified tyrosyl residues do not participate in catalysis directly, but that their effect on activity is indirect.

An increasing body of experimental evidence indicates that the entire structure of an enzyme may decisively affect the interaction between the active site and substrate. Charge-relay systems such as reported in seryl proteases (Blow *et al.*, 1969) and regulatory (Monod *et al.*, 1963) or catalytic mechanisms involving conformational changes of the enzyme (Koshland and Neet, 1968; Christen, 1970; Christen and Riordan, 1970) imply that amino acid residues can affect the rate of the catalytic reaction even though they are not involved directly in bond breaking and making steps.

In the present case, environmental effects of the tyrosyl derivatives on other functional groups may represent one of the many conceivable mechanisms for the increase in activity. However, charge effects of the modified tyrosyl residues are unlikely since the increase in rate does not correlate with the pK of the various tyrosyl derivatives. Alternatively, the modification may lead to subtle conformational changes in the enzyme, perhaps altering subunit interactions. Such a view is consistent with the observation of negative homotropic interactions in substrate binding by alkaline phosphatase: this property would appear to impede substrate binding, and, hence, catalysis, at the second active site of the native enzyme, consequent to the binding of substrate to the first site (Levitzki and Koshland, 1969; Simpson, 1969; Lazdunski *et al.*, 1969; Simpson and Vallee, 1970). Phosphate binding studies have suggested that formation of the aminotyrosyl enzyme largely eliminates the negative interaction between the two active sites (Simpson and Vallee, 1970). Abolition of the negative cooperativity by formation of the aminotyrosyl enzyme should lead to increased apparent activity, both for transfer and hydrolytic reactions. It would not be expected, however, to alter the ratio of these two activities. Hence, while an "allosteric densensitization" may be the basis for part of the observed activity changes, the cause of the overall effect is obviously more complex.

Kinetic investigations have indicated that the rate-limiting step in the reaction of alkaline phosphatase is a conformational change occurring between the formation of the Michaelis complex and the phosphorylation of the enzyme (Halford *et al.*, 1969). The hypothesis of such a rate-limiting isomerization step is supported by the fact that all phosphate esters are hydrolyzed at the same rate regardless of the nature of the leaving group (Heppel *et al.*, 1962). An increase in enzymatic activity such as observed with aminotyrosyl-, iodo-, or *N*-bromosuccinimide-treated phosphatase might reflect a rate enhancement of this isomerization step. Remarkably, the hydrolase activity of aminotyrosyl phosphatase at low ionic strength is increased by about the same factor for all phosphate ester substrates examined (Table V). These data again are consistent with the supposition that the overall rate enhancement in aminotyrosyl phosphatase reflects an increase in rate of the rate-limiting isomerization step common to the reaction of all substrates. Kinetic studies of alkaline phosphatase modified variously in this system of sequential modifications are now being pursued in an effort to relate the effect of the modification of nonactive-site tyrosyl residues to the detailed features of the catalytic reaction of this enzyme.

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Mechanism of Action of Isocitrate Lyase from *Pseudomonas indigofera**

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ABSTRACT: Steady-state kinetic analyses were carried out with highly purified isocitrate lyase from *Pseudomonas indigofera* on both the forward and reverse reactions at pH 6.8 and 7.7. The effect of the inhibitors phosphoenolpyruvate, itaconate, and maleate was also examined. At pH 7.7 the product glyoxylate was found to be a linear competitive inhibitor of isocitrate cleavage, while succinate (at concentrations as high as twice its Michaelis constant) was a linear noncompetitive inhibitor, and itaconate was a linear uncompetitive inhibitor. Phosphoenolpyruvate, itaconate, and maleate were all linear competitive inhibitors with respect to succinate and linear uncompetitive inhibitors with respect to glyoxylate. Kinetic analysis of the data suggests that the binding of glyoxylate and succinate to the enzyme is preferentially ordered with the binding of glyoxylate greatly enhancing succinate binding. Phosphoenolpyruvate, itaconate, and maleate are bound to the succinate-specific site, and require prior binding of glyoxylate.

The equilibrium constant for the condensation reaction at 30° and pH 7.7 was found to be 430 M^{-1} and is in reasonable accord with the value of 630 M^{-1} that was calculated by the Haldane equation.

The mechanism of action and regulation of activity of isocitrate lyase is of particular interest because it is the key enzyme in the anaplerotic glyoxylate cycle, which functions in numerous microorganisms and fatty seedlings (Kornberg and Elsden, 1961; Beevers, 1961). In bacteria its activity is probably important in regulating the intracellular concentrations of tricarboxylic acid cycle intermediates.

Kinetic studies of condensation of succinate and glyoxylate were carried out by Daron *et al.* (1966) on isocitrate lyase from *Pseudomonas aeruginosa*, and they concluded that the reaction proceeded either by a strictly ordered addition of substrates or by random binding of substrates in rapid equilib-

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