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Syncatalytic Modification of a Functional Tyrosyl Residue in Aspartate Aminotransferase*

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ABSTRACT: Chemical modification of enzymes in the presence of substrate, a procedure generally intended to *prevent* reaction of functional amino acid residues, has now been employed to *promote* the reactivity of certain residues of aspartate aminotransferase during the transamination reaction. In the presence of the substrate pair, glutamate and α-ketoglutarate, tetranitromethane abolishes the activity of this enzyme within 1 hr, concomitant with the nitration of one tyrosyl residue. However, in the absence of substrate, neither the pyridoxal 5′-phosphate nor the pyridoxamine 5′-phosphate form of the enzyme is inactivated. In the presence of competitive inhibitors, or of either substrate alone, only slight inactivation occurs. The inactive nitroenzyme is found to be in the pyridoxamine 5′-phosphate form, suggesting that nitration probably occurs during or after the transition of the enzyme–substrate complex from the aldimine to the ketimine intermediate. The quasi-substrate, α-methylaspartate, which does not form a ketimine, does not induce rapid inactivation, thus supporting this view. The susceptibility of the functional tyrosyl residue toward nitration is greatly enhanced, during the catalytic process, relative to that of model compounds seemingly reflecting a short-lived change in its environment. This *syncatalytic*, *i.e.*, synchronous with the catalytic process, activation would appear to be related to the role of this tyrosyl residue in the mechanism of transamination.

Current theories of enzyme action emphasize the importance of transient conformational changes to the overall catalytic process (Koshland and Neet, 1968; Eigen and Hammes, 1963; Hammes, 1968; Ivanov and Karpeisky, 1969). Such changes are thought to facilitate intramolecular interactions between functional groups of the enzyme, to properly align them with groups of the substrate, to induce bond strain, and to underlie allosteric regulation. These theories have been supported by considerable indirect and direct physicochemical evidence, though the latter in an as yet limited number of systems.

We have now found chemical indications that conformational changes of aspartate aminotransferase occur during the transamination reaction. A functional tyrosyl residue, unreactive in the absence of substrates, becomes unusually susceptible to chemical modification when *transamination is actually proceeding*. This change in chemical properties synchronous with catalysis, *i.e.*, syncatalytic,[1] has been detected by reaction with tetranitromethane. The increased reactivity of the essential tyrosyl residue appears only during a particular segment of the catalytic pathway, *i.e.*, during or after the transition from the aldimine to the ketimine, and likely reflects its role in the mechanism of action of the enzyme. A preliminary report of these investigations has been presented (Christen and Riordan, 1969).

Materials

Aspartate aminotransferase (EC 2.6.1.1., cytoplasmic enzyme from pig heart) prepared according to Jenkins *et al.* (1959) was obtained from Boehringer Mannheim Corp. (batch 6308221) as a suspension in 3 M ammonium sulfate–0.05 M maleate–0.0025 M α-ketoglutarate. The specific activity was 440 μ/mg (μmoles of oxalacetate per min per mg) when assayed as described below. Malate dehydrogenase and α-ketoglutaric acid were purchased from Worthington Biochemical Corp.; NADH, L-aspartic acid, pyridoxal 5′-phos-

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[1] The term "syncatalytic" is employed to refer to those events which occur *while catalysis is actually proceeding*, *i.e.*, synchronous with the catalytic process.

phate, pyridoxamine 5′-phosphate, DL-α-methylaspartate, and *N*-acetyl-L-tyrosine ethyl ester from Sigma Chemical Co.; L-glutamic acid, glutaric acid, maleic acid, phthalic acid, and 2-mercaptoethanol from Eastman Organic Chemicals; L-alanine from Ajinomoto Co., Tokyo; sodium hydrosulfite from Fisher Scientific Co.; *N*-acetyl-L-tyrosine from Cyclo Chemical Corp.; *N*-AcTyr-NH_2 from Mann Research Laboratories; tetranitromethane from Aldrich Chemical Co. *p*-Mercuribenzoate was obtained from Sigma Chemical Co. and was precipitated four times before use (Boyer, 1954). Alkaline phosphatase from *E. coli* was prepared according to Simpson *et al.* (1968).

Methods

Aspartate aminotransferase (about 5 mg/ml) was dialyzed prior to use against a 100-fold volume of 0.05 M Tris-Cl (pH 7.5) at 4°. The dialysate was changed three times with intervals of at least 6 hr. Enzyme concentrations were determined from the absorbance at 280 nm, using a molar absorptivity of ϵ_{280} 2.1×10^5. This value is derived from the absorbance at 388 nm in 0.1 N NaOH, assuming the molar absorptivity of pyridoxal 5′-phosphate, ϵ_{388} 6600 (Peterson and Sober, 1954). Molar enzyme concentrations were converted into milligrams per milliliter on the basis of a molecular weight of 78,600 (Banks *et al.*, 1968).

Transaminase activity was determined according to Karmen (1955) using a Unicam SP-800 recording spectrophotometer thermostatted at 25°. The assay mixture consisted of 0.075 M potassium phosphate, 0.2 M L-aspartate, 0.0085 M α-ketoglutarate, 0.2 mM NADH, and 50 μg of malate dehydrogenase (pH 7.6) in a final volume of 3.0 ml. The preparation of malate dehydrogenase used exhibited a small NADH oxidizing activity. The assays were carried out at concentrations of aspartate aminotransferase sufficient to result in an activity at least 20 times higher than this blank activity and were corrected.

The pyridoxal 5′-phosphate form of aspartate aminotransferase (5 mg/ml) was converted into the pyridoxamine 5′-phosphate form by dialysis against a 50-fold volume of 0.1 M potassium phosphate–0.25 M aspartate–0.2 mM NADH (pH 7.5) containing 0.5 mg of malate dehydrogenase. After 1 hr at 4° the enzyme was dialyzed further against three changes of a 250-fold volume of 0.05 M Tris-Cl (pH 7.5).

Apoenzyme was prepared following the procedure of Scardi *et al.* (1963). Glutamate was added to holoenzyme in 0.05 M Tris-Cl (pH 8.0) to a final concentration of 0.1 M. After 15 min at room temperature the enzyme solution was dialyzed against a 200-fold volume of 0.5 M potassium phosphate (pH 5.0) for 2 hr at 30° and subsequently dialyzed at 4° against three changes of a 100-fold volume of 0.05 M Tris-Cl at the desired pH.

The coenzyme was isolated from the enzyme using the procedure of Jenkins and Sizer (1960). The enzyme solution was made 0.1 N in NaOH and the protein was then precipitated at 0° by trichloroacetic acid, final concentration 6%. After centrifugation the precipitate was washed twice with 5% trichloroacetic acid. The first supernatant was pooled with the two wash solutions and extracted with ether until neutral. The yield of coenzyme was 60–70%. P_i was determined by the procedure of Chen *et al.* (1956).

Nitrotyrosyl content was determined spectrophotometrically at pH 8.5 using an ϵ_{428} value of 4100 (Sokolovsky *et al.*, 1966). In the case of holoenzyme A_{428} was corrected for coenzyme absorption.

Sulfhydryl groups were determined with *p*-mercuribenzoate (Boyer, 1954). The reagent was dissolved in 0.1 M NaOH and then diluted with 0.25 M potassium phosphate (pH 7.0). Its concentration was determined by measuring A_{232} and a 15-fold molar excess was added to 2 ml of a 5×10^{-6} M solution of protein in 0.2 M potassium phosphate (pH 7.0). The reported numbers of sulfhydryl groups titrated are based on ΔA_{250} read after 1 hr at room temperature. After this period A_{250} increased at identical and very slow rates in all samples.

Absorption at discrete wavelengths was determined with a Zeiss PMQ II spectrophotometer. Absorption spectra were obtained with a Cary Model 15 spectrophotometer. Spectral titrations were performed with a pH–titration cell (Auld and French, 1970). Circular dichroism was measured with a Cary Model 60 spectropolarimeter equipped with the Model 6001 circular dichroism attachment. Sedimentation velocity was determined with a Spinco Model E ultracentrifuge. Chromatographic analyses for nitrotyrosyl content were performed with a Spinco Model 120C amino acid analyzer. The samples were hydrolyzed in sealed, evacuated tubes in 6 N HCl, containing 10 μl of phenol/ml at 110° for 22 hr. In order to obtain a sizable nitrotyrosyl peak, an amount of hydrolysate corresponding to 0.05 μmole of protein was applied to the long column. Chromatography was carried out with 0.2 M sodium citrate buffer (pH 4.3) and the ninhydrin reagent was mixed with the column effluent 40 min after starting the run. Cellulose-precoated Eastman Chromagram sheets, "6065 cellulose with fluorescent indicator," were used for thin-layer chromatography of the coenzymes. The solvent system, *t*-butyl alcohol–formic acid–water (70:15:15, v/v) (Cattanéo *et al.*, 1960) gave good separation of pyridoxamine, pyridoxal, pyridoxamine 5′-phosphate, and pyridoxal 5′-phosphate.

Results

Reaction of Aspartate Aminotransferase with Tetranitromethane in the Presence and Absence of Substrates. The presence of substrates in the reaction mixture markedly alters the effects of tetranitromethane on the enzymatic activity of aspartate aminotransferase (Figure 1). In the presence of the substrate pair, glutamate and α-ketoglutarate, the enzyme is inactivated rapidly and virtually completely.[2] In the presence

[2] The catalysis-dependent inactivation of aspartate aminotransferase by tetranitromethane consistently results in a residual activity of 2–4% after a reaction time of 1 hr (see Figure 1). Even if more tetranitromethane is added after 1 hr this residual activity decreases only very slowly, the presence of the substrate pair again accelerating the reaction though to a lesser degree than before. Only a slight change in the spectrum of an enzyme solution with a residual activity of 3% is observed on addition of a fivefold molar excess of α-ketoglutarate (final concentration 0.15 mM): a decrease at 330 nm, 2% of the total A_{330}, is accompanied by an increase in A_{360}, about 5% of the total A_{360}. Similarly no marked spectral changes are observed after dialysis of the inactivated enzyme against two changes of 5 mM α-ketoglutarate. Thus, the residual activity is probably due to a contaminating pyridoxal 5′-phosphate dependent enzyme with transaminase activity rather than to a true residual activity of inactivated aspartate aminotransferase. This is further confirmed by a single turnover assay. Nitrated enzyme (residual activity 3%, 2.0 nitrotyrosyl residues/molecule, final concentration

TABLE I: Reaction of Aspartate Aminotransferase with Tetranitromethane in the Presence and Absence of Substrates: Amino Acid Modifications.[a]

Glutamate and α-Keto-glutarate	Tyrosyl Residues Nitrated[b] (moles/mole)		SH Groups Oxidized (moles/mole)		% Inactivation	
	1 hr	2 hr	1 hr	2 hr	1 hr	2 hr
A. Present	1.7	2.4	1.7	1.9	97	98
B. Absent	0.7	1.2	1.4	1.5	5	8
A − B	1.0	1.2	0.3	0.4	92	90

[a] Conditions are those of Figure 1. The reaction was stopped after 1 or 2 hr, respectively, by addition of 2-mercaptoethanol (0.125 M). Identical results were obtained when the reaction was quenched with phenol (5 mM) or by cooling to 4° and subsequent dialysis. Activity and nitrotyrosyl content were measured after dialysis at 4° against three changes of a 100-fold volume of 0.05 M Tris-Cl (pH 8.5). Sulfhydryl groups were measured using *p*-mercuribenzoate. The data are given as the differences between the native and modified enzymes. Native aspartate aminotransferase was found to have about six titratable sulfhydryl groups and 24 tyrosyl residues (Martinez-Carrion *et al.*, 1967). [b] Nitrotyrosyl content as determined by spectral analysis. Amino acid analyses gave 2.3 and 0.2 nitrotyrosyl residues per molecule, at 1 hr, respectively. Since the enzyme contains 46 phenylalanyl residues per mole (Martinez-Carrion *et al.*, 1967) maximally 0.05 μmole of hydrolyzed enzyme could be applied to the column without lifting appreciably the base line in the elution region of nitrotyrosine. This small load of nitrotyrosine resulted in only semiquantitative agreement with the values derived from spectral data.

of only one substrate, *i.e.*, either glutamate or α-ketoglutarate, the enzyme is inactivated slowly. In the absence of substrates, neither the pyridoxal 5′-phosphate nor the pyridoxamine 5′-phosphate form of the enzyme is inactivated appreciably.

Nature of the Essential Modified Group. The inactivation appears to be due to the catalysis-dependent nitration of a particular tyrosyl residue (Table I). In the presence and absence of substrates both tyrosyl and sulfhydryl residues are modified, consistent with the reported specificity of tetranitromethane (Sokolovsky *et al.*, 1966; Riordan and Christen, 1968). However, substrate-induced inactivation coincides with the additional nitration of 1 tyrosyl residue (Figure 2) and the loss of only 0.3 sulfhydryl group/molecule of enzyme. The degree of inactivation and of nitration and the loss of titratable sulfhydryl groups are the same when the reaction is stopped either by cooling to 4°, or by addition of 2-mercaptoethanol or of phenol.

7×10^{-6} M) was dialyzed against 5 mM α-ketoglutarate and then against three changes of 0.05 M Tris-Cl (pH 7.5). NADH (final concentration 0.2 mM) and malate dehydrogenase (30 μg) were added to give a final volume of 1.5 ml. The change in A_{340} following the addition of 15 μl of 1 M aspartate was recorded with a solution without aspartate but otherwise of identical composition in the reference beam. The observed decrease in A_{340} did not exceed the dilution effect on addition of the aspartate solution (10% of that which would be expected if 2 moles of NADH/mole of enzyme was oxidized).

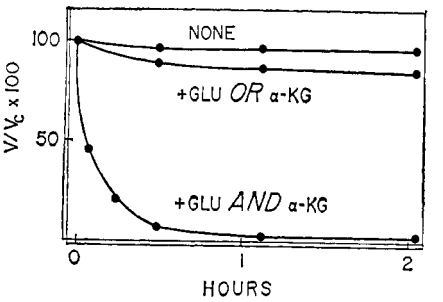


FIGURE 1: Catalysis-dependent inactivation of aspartate aminotransferase by tetranitromethane. Enzyme, 2.1×10^{-6} M, in 0.05 M Tris-Cl (pH 7.5) was incubated at 22° with 6.0×10^{-4} M tetranitromethane in the presence of both 70 mM L-glutamate (GLU) *and* 1.75 mM α-ketoglutarate (αKG), in the presence of either glutamate *or* α-ketoglutarate, and in the absence of either substrate. Tetranitromethane was added at zero time and at the indicated times transaminase activity was determined. The substrate concentrations are 17.5 times the values of the respective Michaelis constants (Velick and Vavra, 1962). The order of addition of enzyme, substrates, and tetranitromethane to the reaction mixture did not influence the course of the reaction.

The Coenzyme after Catalysis-Dependent Inactivation. The absorption spectrum of inactive aminotransferase, nitrated in the presence of substrates, exhibits a nitrotyrosine absorption spectrum with a maximum at 428 nm and another at 330 nm due to bound coenzyme (Figure 3). Comparison with the native pyridoxamine 5′-phosphate enzyme suggests that the coenzyme moiety of the inactivated enzyme exists entirely in the form of pyridoxamine 5′-phosphate. Compared with the native enzymes, the spectra of both the pyridoxal 5′-phosphate and the pyridoxamine 5′-phosphate enzymes (not shown in Figure 3), incubated with tetranitromethane in the absence of substrates, are unchanged in the region of coenzyme absorption.

Further, as is characteristic for the pyridoxamine 5′-phosphate form (Jenkins and Sizer, 1960), the spectrum of the

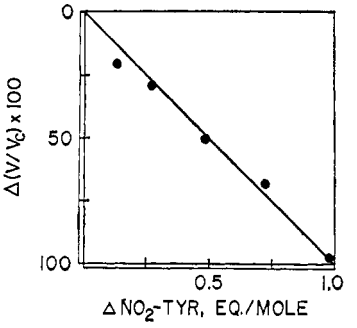


FIGURE 2: Catalysis-dependent inactivation of aspartate aminotransferase and tyrosyl nitration by tetranitromethane. Conditions are those of Figure 1. After different times the reactions were stopped by addition of 2-mercaptoethanol. Activity and nitrotyrosyl content were determined as in Table I. The activity of enzyme incubated with tetranitromethane in the presence of substrates minus that of enzyme incubated with tetranitromethane alone is plotted *vs.* the difference in nitrotyrosyl content of the same samples.

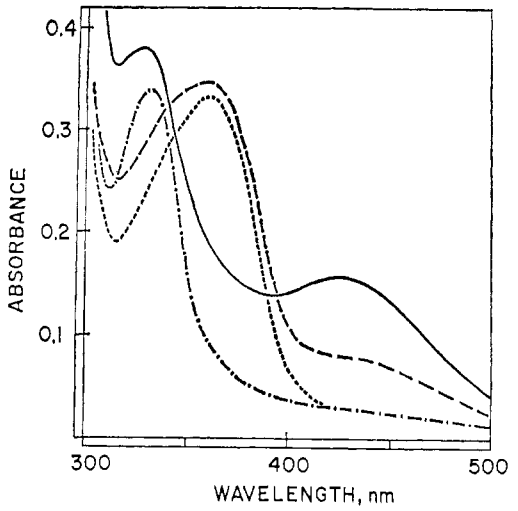


FIGURE 3: Absorption spectra of aspartate aminotransferase nitrated in the presence and absence of substrates and native pyridoxamine 5′-phosphate and pyridoxal 5′-phosphate enzyme. Conditions for nitration were those of Table I. The enzyme concentration is 1.8×10^{-5} M in 0.05 M Tris-Cl (pH 8.5); incubated with tetranitromethane in the presence of glutamate and α-ketoglutarate (——), residual activity 3% of control, 1.7 moles of nitrotyrosine/mole; pyridoxal 5′-phosphate enzyme incubated with tetranitromethane in the absence of substrates (– –), residual activity 93% of control, 0.7 mole of nitrotyrosine/mole; native pyridoxamine 5′-phosphate enzyme (–·–); native pyridoxal 5′-phosphate enzyme (----).

TABLE II: Comparison of Cofactor Isolated from Inactivated Aminotransferase with Pyridoxamine 5′-Phosphate.

	λ_{max} (ε)		Activation of Apoaminotransferase[a] $v/v_{native} \times 100$
	pH 2	pH 12	
Cofactor[b]	293 (9150)[c]	306 (7200)[c]	66
Pyridoxamine 5′-phosphate	293 (9000)[d]	308 (6700)[d]	78

[a] Apoenzyme with a residual activity of 6% was prepared as described under Methods. A twofold molar excess of authentic pyridoxamine 5′-phosphate or of the isolated coenzyme was added to a 2.2×10^{-5} M solution of apoenzyme in 0.05 M Tris-Cl (pH 8.5). Activities were determined after 2 hr at room temperature. [b] Aminotransferase was inactivated by tetranitromethane in the presence of substrates as in Table I and then dialyzed against 0.05 M Tris-Cl (pH 8.5). The product had a residual activity of 2% and contained 1.7 nitrotyrosyl residues/molecule. The coenzyme was isolated as described under Methods. [c] The molar absorptivities are based on determinations of P_i after incubation of the isolated cofactor with alkaline phosphatase at pH 8–9. [d] Values from Peterson and Sober (1954).

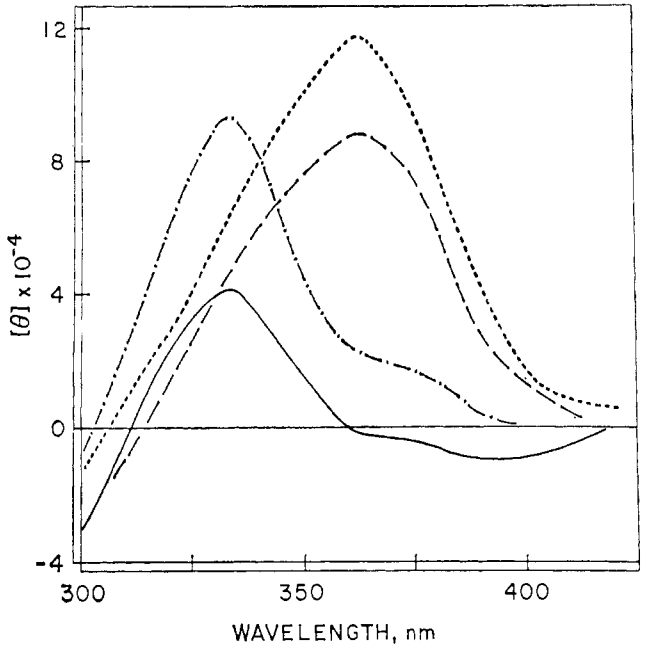


FIGURE 4: Circular dichroic spectra of aspartate aminotransferase nitrated in the presence and absence of substrates and of native pyridoxamine 5′-phosphate and pyridoxal 5′-phosphate enzyme. Conditions and notations are the same as in Figure 3: nitrated in the presence of the susbstrate pair (——). (The basis for the decrease in amplitude and for the newly generated Cotton effect near 395 nm is unclear and remains to be elucidated); pyridoxal 5′-phosphate enzyme nitrated in the absence of substrates (– –); native pyridoxamine 5′-phosphate enzyme (–·–); native pyridoxal 5′-phosphate enzyme (----). Enzyme concentrations were 2×10^{-5} M in 0.05 M Tris-Cl (pH 8.5) in a 1-cm cell.

inactivated enzyme is not changed by lowering the pH to 4.7. In contrast, the spectrum of the pyridoxal 5′-phosphate enzyme treated with tetranitromethane alone exhibits a shift of the maximum from 362 to 430 nm under these conditions.

The cofactor bound to the inactivated enzyme has been isolated. Its λ_{max} and ε in acid and in base are essentially identical with those of authentic pyridoxamine 5′-phosphate (Table II). Further, the isolated material is capable of reactivating inactive apoenzyme to the same extent as authentic pyridoxamine 5′-phosphate (Table II), and it migrates with the same R_F as pyridoxamine on thin-layer chromatography.[3]

The absorption band centered at 428 nm (Figure 3) originates from the protein rather than from the coenzyme. It persists when the coenzyme is separated from the protein either by the method of Scardi *et al.* (1963) or by alkalinization and subsequent precipitation with trichloroacetic acid (Jenkins and Sizer, 1960). Spectral titration (in 4 M urea to prevent precipitation) gives an apparent pK of 7.3 close to the value of 7.2 reported for 3-nitrotyrosine (Sokolovsky *et al.*, 1967). Treatment of the inactivated enzyme with 5 mM sodium hydrosulfite abolishes the band at 428 nm without affecting the coenzyme absorption, reflecting the reduction of 3-nitrotyrosyl residues (Sokolovsky *et al.*, 1967). Reduction does not alter the activity of the modified enzyme.

[3] Authentic pyridoxamine 5′-phosphate, when subjected to the procedure for isolating the cofactor from the protein and subsequent to the rotary evaporation step for concentrating the samples, migrated with the same R_F as pyridoxamine indicating that hydrolysis occurred during concentration of the samples.

TABLE III: Reactivation of Resolved Enyzme Derivatives by Pyridoxal 5′-Phosphate and Pyridoxamine 5′-Phosphate.[a]

Enzyme Derivative	Relative Activity			
	Before Resolution	After Resolution[b]		
		No Addn	+ PLP[c,d]	+ PMP[c,d]
Native	100	1	62	48
Incubated with tetranitromethane[a]	92	2	62	35
Incubated with tetranitromethane + glutamate and α-ketoglutarate[a]	4	0.2	3	3

[a] Prepared as in Table I. [b] Resolution as described under Methods. [c] The samples (about 2×10^{-5} M) were reconstituted by incubation with a twofold molar excess of coenzyme for 1 hr, 4°. [d] PLP = pyridoxal 5′-phosphate; PMP = pyridoxamine 5′-phosphate.

TABLE IV: Sedimentation Coefficients of Native and Modified Aspartate Aminotransferase.

Enzyme Derivative	s^{20} (S)	Rel Act. % Control
Native	5.7	100
Incubated with tetranitromethane[a]	5.6	93
Incubated with tetranitromethane + glutamate and α-ketoglutarate[a]	5.6	3

[a] Prepared as in Table I. Centrifugation was in 0.05 M Tris-Cl (pH 8.5) at a protein concentration of about 2.5×10^{-5} M (2.0 mg/ml).

Characteristics of the Inactivated Enzyme. Since the coenzyme of the inactivated enzyme is present almost exclusively in the pyridoxamine 5′-phosphate form (Figure 3) it would seem that syncatalytic nitration prevents conversion of the pyridoxamine 5′-phosphate into the pyridoxal 5′-phosphate form. In fact, exhaustive dialysis of the inactive enzyme against 5 mM α-ketoglutarate neither abolishes the absorption at 330 nm nor regenerates the 362-nm band. Apparently, the coenzyme is trapped irreversibly in the pyridoxamine form.[2]

The circular dichroic spectrum of the inactivated enzyme indicates that neither the coenzyme nor its asymmetric mode of binding have been affected by the modification (Figure 4). The inactivated enzyme retains the positive Cotton effect at 333 nm typical of the native pyridoxamine 5′-phosphate form of aminotransferase.

The inactive nitrotyrosyl enzyme, when resolved, will not rebind coenzyme as evidenced by the lack of a significant change in the absorption spectrum upon addition of either pyridoxal 5′-phosphate or pyridoxamine 5′-phosphate. Moreover, neither form of the coenzyme will reactivate the aponitrotyrosyl enzyme (Table III).

Circular dichroism measurements and sedimentation analysis have not detected gross structural alterations in the modified protein. The circular dichroic spectrum of native and inactivated enzyme appear to be identical at wavelengths below 250 nm. The molecular ellipticities at the minimum centered at 218 nm are 2.6×10^7 and 2.7×10^7 for the native and the inactivated enzyme, respectively. The apparent sedimentation coefficients of both enzyme derivatives incubated with tetranitromethane in the presence or in the absence of the substrate pair do not differ significantly from that of the native enzyme (Table IV).

Nitration of tyrosine shifts the phenolic pK' from 10.1 (Edelhoch, 1962) to 7.2 (Sokolovsky *et al.*, 1967). If the phenolic hydroxyl group were involved in activity, nitration might shift the pH–rate profile and lead to apparent inactivation at a particular value of pH. However, the same residual activity is measured at pH 6.5, 7.6, and 8.5. Reduction of the 3-nitrotyrosyl to a 3-aminotyrosyl residue (see above) also does not alter activity though the pK' of the phenolic hydroxyl group of 3-aminotyrosine is 10.0 (Sokolovsky *et al.*, 1967), close to that of tyrosine.

Catalysis-Linked Reactivity Changes of the Essential Tyrosyl Residue. The reactivity of the essential tyrosyl residue in the various intermediates of the catalytic reaction (see equation) has been studied using single substrates, competitive inhibitors and substrate analogs. The pyridoxamine 5′-phosphate enzyme in the presence of glutamate and the pyridoxal 5′-phosphate enzyme in the presence of α-ketoglutarate are inactivated very slowly by tetranitromethane (Table V). Binding of competitive inhibitors also fails to induce rapid inactivation with both the pyridoxal 5′-phosphate and the pyridoxamine 5′-phosphate forms of the enzyme and, in fact, protects the enzyme from inactivation by tetranitromethane in the presence of the substrate pair (Figure 5). An inhibitor concentration of 10 times its dissociation con-

TABLE V: Inactivation of Aspartate Aminotransferase by Tetranitromethane in the Presence of Substrates and Competitive Inhibitors.[a]

Addition	$v/v_c \times 100$	
	Pyridoxal 5′-Phosphate Enzyme	Pyridoxamine 5′-Phosphate Enzyme
Glutamate	83[b]	73
α-Ketoglutarate	88	72
Maleate	91	
Glutarate	89	92
Phthalate	89	
None	96	81

[a] Conditions were as in Figure 1. Activities were measured after 1 hr. Concentrations of maleate, glutarate, and phthalate are 45, 60, and 100 mM, respectively (Velick and Vavra, 1962). [b] At low enzyme concentration (4×10^{-7} M).

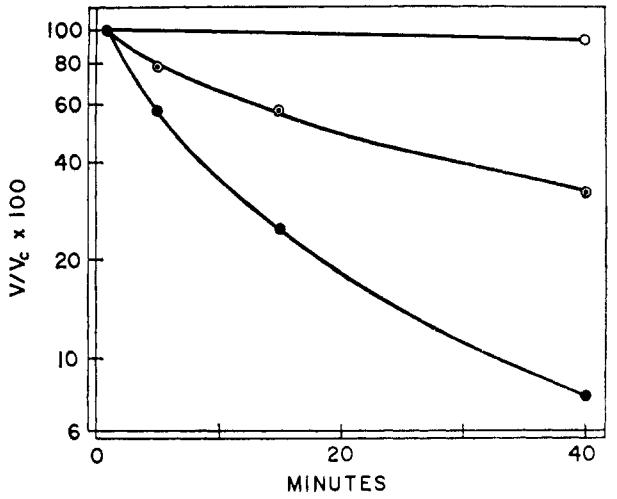


FIGURE 5: Protection by a competitive inhibitor against catalysis-dependent inactivation by tetranitromethane. Conditions are the same as those of Figure 1, reaction mixture containing no substrate (○); the substrate pair (each at 17.5 × K_m) (●); and the substrate pair plus 50 mM phthalate (10 × K_d) (⊙).

TABLE VI: Activity, Nitrotyrosyl, and Sulfhydryl Content of Aspartate Aminotransferase Incubated with Tetranitromethane in the Presence and Absence of α-Methylaspartate.[a]

	% Inactivation	Nitrotyrosyl Residues/Molecule	SH Groups Oxidized/Molecule
A. Incubated with tetranitromethane + α-methylaspartate	93	3.9	2.6
B. Incubated with tetranitromethane	22	2.2	1.4
A − B	71	1.7	1.2

[a] Pyridoxal 5′-phosphate enzyme was incubated with 1.2 × 10^{-3} M tetranitromethane (2× the usual concentration). Reaction mixture A contained 0.15 M DL-α-methylaspartate. The reaction was stopped after 2 hr with 0.125 M 2-mercaptoethanol. Other conditions were as in Figure 1.

stant (Velick and Vavra, 1962) decreases the rate of inactivation about twofold. Thus, of itself the formation of adsorption (Michaelis) complexes seems insufficient to induce reactivity toward tetranitromethane leaving the aldimine or ketimine as the most probable tetranitromethane-reactive intermediates.

The substrate analog, α-methylaspartate, induces some enhancement in the rate of inactivation, but this rate is still 15 times slower than that observed in the presence of the substrate pair when aldimines and ketimines are both formed (Figure 6). However, with twice the concentration of tetranitromethane, the enzyme in the presence of α-methylaspartate is inactivated to 7% of its initial activity within 2 hr (Table VI). Under these conditions enzyme incubated with tetranitromethane alone retains 78% of its activity. Inactivation is still accompanied by tyrosyl nitration.

In order to assess the reactivity of the various intermediates, the rates of reaction with tetranitromethane are compared with those of tyrosyl model compounds (*N*-acetyltyrosine, *N*-acetyltyrosine ethyl ester, and *N*-acetyltyrosinamide) (Table VII). Inactivation of the enzyme in the absence of any substrate is markedly slower than reaction of the tyrosyl model compounds. In the presence of α-methylaspartate, when aldimine intermediates are formed, the essential tyrosyl residue is nitrated at a rate comparable with that of the model. However, in the presence of the substrate pair, when ketimine intermediates are formed, the rate of nitration is increased 15 times over that of tyrosine itself.

The rates of enzyme inactivation and of the reaction of tetranitromethane with tyrosine model compounds were determined as a function of pH (Figure 7). Under all conditions, with or without substrates, or with α-methylaspartate, the rates of inactivation, *i.e.*, of nitration of the essential tyrosyl residue, are largely independent of pH. This con-

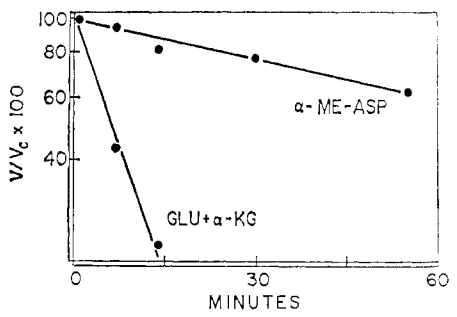


FIGURE 6: Inactivation of aspartate aminotransferase with tetranitromethane. Comparison of the effect of 0.14 M DL-α-methylaspartate (α-Me-ASP) with that of glutamate plus α-ketoglutarate. Conditions were those of Figure 1.

TABLE VII: Rates of Reaction with Tetranitromethane.[a]

Reactant	Rate Constant k_{app} × 10, min^{-1}
Tyrosine model compounds[b]	0.06
Aspartate aminotransferase	
Native	0.01[c]
In the presence of α-methylaspartate (aldimine complex)	0.08[c]
In the presence of glutamate and α-ketoglutarate (aldimine and ketimine complexes)	1.2[c]

[a] Conditions were 2.1 × 10^{-5} M reactant and 0.6 mM tetranitromethane in 0.05 M Tris-Cl (pH 7.5) at 22°. [b] *N*-Acetyltyrosine, *N*-acetyltyrosine ethyl ester, and *N*-acetyltyrosinamide. The rate of nitroformate production was measured spectrophotometrically, ϵ_{350} 14,400 (Glover and Landsman, 1964). [c] Rate of inactivation; conditions were those of Figures 1 and 6, respectively.

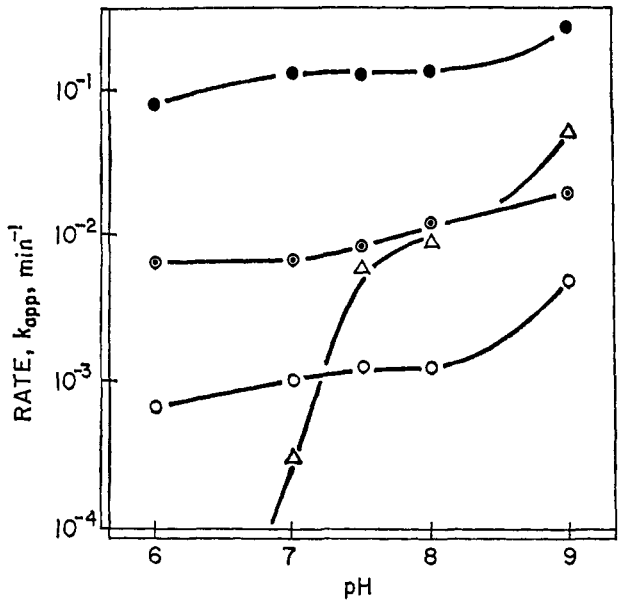


FIGURE 7: The pH dependence of inactivation of aspartate aminotransferase by tetranitromethane and reaction of tyrosyl model compounds with tetranitromethane. Conditions are those of Table VII. At pH 7, 8, and 9 the buffer was Tris-Cl and at pH 6 it was Tris-acetate. Rate of enzyme inactivation in the presence of glutamate and α-ketoglutarate (●), in the presence of α-methylaspartate (⊙), and in the absence of substrate (○); rate of nitroformate production with tyrosyl model compounds (△) (*cf.* legend of Table VII). Inactivation at pH 6 also correlated with the nitration of one extra tyrosyl residue indicating that over the entire pH range the rate of inactivation in the presence of substrates may be equated with the rate of nitration of the functional tyrosyl residue. *N*-Acetyltyrosine, *N*-acetyltyrosine ethyl ester, and *N*-acetyltyrosin amide served as the models and were all nitrated at about the same rate.

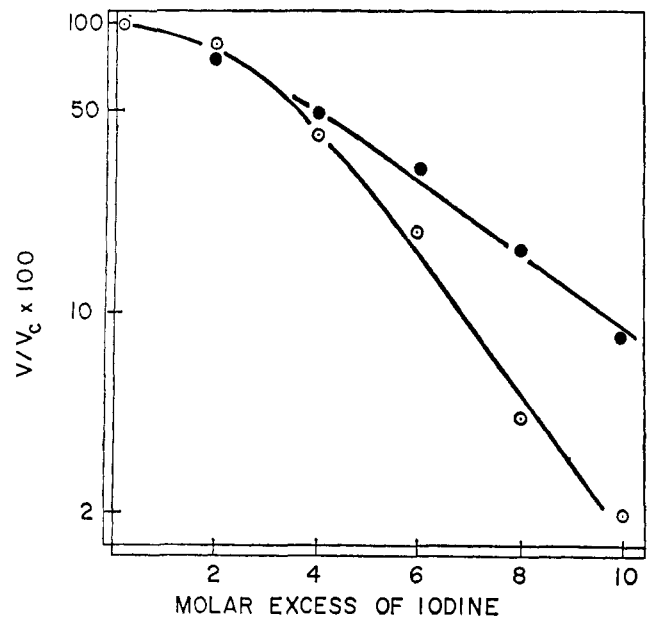


FIGURE 8: Inactivation of aspartate aminotransferase by iodine in the presence (⊙) and absence (●) of substrates. Aliquots of 0.05 M iodine in 0.5 M potassium iodide were added successively to give the molar excesses indicated. Activity was determined 3 min after each addition of iodine. Other conditions were as in Figure 1.

trasts with the rates of reaction of tetranitromethane with the tyrosyl model compounds which are markedly pH dependent.

Syncatalytic activation is also detected with other probing reagents. Qualitatively, the same positive effect of substrates is observed on the rate of inactivation with iodine, though this reaction is complicated both by the fact that the enzyme is inactivated even in the absence of the substrates and also by apparent initial substrate protection. However, a catalysis-dependent component of the inactivation in the presence of substrates is clearly discernible (Figure 8).

Discussion

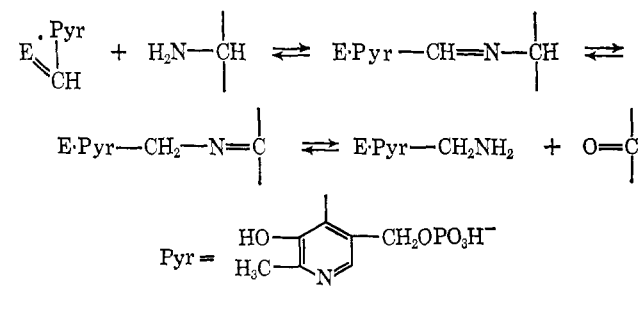
A number of studies have indicated that the transamination reaction catalyzed by aspartate aminotransferase involves multiple enzyme–substrate intermediates (Snell, 1962; Braunstein, 1964; Meister, 1965; Fasella, 1968; Hammes and Haslam, 1969; Ivanov and Karpeisky, 1969). The essential features of this reaction are shown in Scheme I.

The pyridoxal 5′-phosphate form of the enzyme reacts with an α-amino acid to give an aldimine which tautomerizes to a ketimine. Subsequent hydrolysis yields the pyridoxamine 5′-phosphate form of the enzyme and the corresponding α-keto acid. Reversal of the reaction with another α-keto acid completes the transamination cycle (Snell, 1962). In the presence of glutamate and α-ketoglutarate, the substrate pair employed in this study, the enzyme will shuttle through the reaction pathway, and an equilibrium concentration of each intermediate will be established in the reaction mixture.

Apparently, in one of these intermediary enzyme–substrate complexes a functional residue, which is unreactive in the absence of substrates, becomes highly susceptible to modification with tetranitromethane.

Consistent with the reported specificity of tetranitromethane (Sokolovsky *et al.*, 1966) tyrosyl and cysteinyl residues are found to be modified. Throughout the entire range of catalysis-induced inactivation, loss of activity correlates linearly with nitration of one tyrosyl residue (Figure 2). Apparently, the modification of this specific residue causes the inactivation and, hence, it would seem that this residue is identical with the group exhibiting catalysis-induced reactivity. Sulfhydryl oxidation can be ruled out as a probable cause of inactivation since in the inactivated enzyme less than 1 group/molecule is oxidized in addition to the 1.4 groups oxidized in the absence of substrate without effect on activity. It has been shown that up to two of the six available sulfhydryl groups can react with *p*-mercuribenzoate

SCHEME I



or Ag^+ without significant loss of activity (Polyanovsky and Torchinsky, 1963). The small additional modification of sulfhydryl groups found here might relate to the observation that a sulfhydryl group is exposed when an amino acid reacts with aspartate aminotransferase (Evangelopoulos and Sizer, 1968.)

Tetranitromethane has been reported to react with tryptophanyl residues in proteins under certain conditions (Cuatrecasas *et al.*, 1968; Sokolovsky *et al.*, 1970). However no significant difference in tryptophan content has been observed between the native enzyme and that treated with tetranitromethane in the presence or absence of substrates. Moreover, since inactivation still occurs at pH 6, this would suggest that tryptophan modification is probably not involved (Sokolovsky *et al.*, 1969). Physicochemical studies have not revealed any gross structural differences between the active and inactive enzymes.

Inactivation is not due to modification of the coenzyme. The spectral and functional characteristics of the cofactor, isolated after separation from the inactivated holoenzyme, are virtually identical with those of authentic pyridoxamine 5′-phosphate (Table II). Further, the spectral properties of the inactive aminotransferase in the region of coenzyme absorption are quite similar to those of the native pyridoxamine 5′-phosphate enzyme. In addition, it does not resemble the inactive γ subform of the enzyme (Martinez-Carrion *et al.*, 1967) since its circular dichroic spectrum indicates that the coenzyme is still bound asymmetrically.

In view of the fact that the coenzyme is not altered by the reaction with tetranitromethane (Table II), examination of its spectral properties could assist in locating the reactive intermediate in the catalytic pathway. The coenzyme of aminotransferase, inactivated by tetranitromethane in the presence of the substrate pair, exists entirely in the pyridoxamine 5′-phosphate form (Figure 3). The modification apparently prevents its conversion into an aldimine or into the pyridoxal 5′-phosphate form. This block of the tautomeric shift together with the fact that *all* coenzyme is found in the pyridoxamine 5′-phosphate form suggests that the highly reactive intermediate occurs during or after the aldimine–ketimine transition (see Scheme I). This view is supported by the fact that α-methylaspartate, a substrate analog which cannot tautomerize to the ketimine (Braunstein, 1964), fails to induce rapid inactivation (Figure 6). Moreover, similar results are obtained on formation of noncovalent enzyme complexes with substrates and competitive inhibitors (Table V, Figure 5). Phthalate, for instance, not only fails to promote inactivation but also protects the enzyme from substrate-induced inactivation (Figure 5).

The changes in the reactivity of the essential tyrosyl residue seem to reflect alterations in its environment in the course of catalysis. Such environmental changes may result either solely from conformational changes of the enzyme–coenzyme–substrate complex during the catalytic process or from direct involvement of the critical group in bond-breaking and -making processes without conformational changes of the protein, or both. However, in the present case, without invoking some topographical reorganization, it is difficult to account for the catalysis-induced abolition of the "buried," unreactive character of the functional residue that preexists the addition of substrate.

The reactivity of tyrosyl residues in simple peptides increases with pH above neutrality (Sokolovsky *et al.*, 1966). In contrast, the reactivity of the functional tyrosyl residue of aminotransferase is remarkably independent of pH (Figure 7). At pH 7.5 the enzyme is nitrated about 20 times and at pH 6.0 about 1000 times faster than the tyrosine models studied. This increase in rate might actually be higher, since at equilibrium only a fraction of the total enzyme will exist as the highly reactive intermediate.

Catalysis-induced inactivation is observed not only with tetranitromethane but also with iodine (Figure 8), indicating that the tetranitromethane reaction reflects activation of a particular group of the enzyme rather than of a specific reagent.

Sedimentation studies as well as the presence of two coenzyme molecules per molecule of enzyme indicate that aspartate aminotransferase is composed of two catalytically active subunits (Fasella, 1968; Banks *et al.*, 1968). However, only about one additional nitrotyrosyl residue per enzyme molecule is nitrated consistently in the presence of the substrate pair correlating with virtually complete inactivation. Concomitantly, both coenzyme molecules per enzyme molecule are converted irreversibly into the pyridoxamine 5′-phosphate form. Based on such stoichiometry it is not clear why both subunits are inactivated on nitration of a single tyrosine. It could be that the 1.7–2.2 nitrotyrosyl residues/molecule found in the inactivated enzyme in fact represent nitration of one functional tyrosyl residue per subunit. The 0.7–1.2 tyrosyl residues, nitrated in the absence of substrates, might become unreactive in the presence of substrate. Such a situation has been observed with the staphylococcal nuclease (Cuatrecasas *et al.*, 1968). Alternatively, this phenomenon could be a manifestation of negative cooperativity, observed recently in a number of enzymes (Conway and Koshland, 1968; Levitzki and Koshland, 1969; Simpson and Vallee, 1970).

Loss of enzymatic activity, consequent to modification of a particular amino acid side chain, is generally thought to indicate the participation of that residue in the catalytic process (Richards, 1959). In the present instance, the syncatalytic nature of the inactivation process is a novel verification of this presumption. This mode of inactivation might reflect the role of this particular residue in catalysis. The maximal catalytic rate of aspartate aminotransferase and the relative proportions of the observable enzyme substrate complexes are reasonably constant between pH 6 and 9 (Velick and Vavra, 1962). Similarly, the reactivity of the nitratable tyrosyl residue is also remarkably independent of pH within the same range (Figure 7), as might be expected if the syncatalytic activation of this residue were functionally significant.

The specific role of syncatalytic activation of the functional tyrosyl residue remains to be elucidated. Apparently the inactivation is not merely due to the shift of the phenolic pK caused by nitration, as has been found with subtilisin (Ottesen *et al.*, 1970) since the nitrated enzyme is inactive from pH 6.5 to 8.5. Further, activity is not restored when the nitrotyrosyl is reduced to an aminotyrosyl residue which has a phenolic hydroxyl pK close to that of tyrosine (Sokolovsky *et al.*, 1967).

On the basis of other lines of evidence, Ivanov and Karpeisky (1969) have proposed a detailed multistage mechanism of action of aspartate aminotransferase that involves a

tyrosyl residue participating in topological alterations of the enzyme–coenzyme–substrate system. Their view of multistage catalysis emphasizes "stabilization-orientation-change of conditions by positional change" as its principal features and is consistent with the observation of syncatalytic side-chain activation, induced by conformational changes. The functional tyrosyl residue implicated by the present studies may or may not be identical with that postulated by these workers.

Turano and collaborators have found that nitration of a tyrosyl residue in apoaspartate aminotransferase prevents recombination with the coenzyme (C. Turano, personal communication). Again, additional experimentation is required to elucidate the relation, if any, of this residue with the one that undergoes syncatalytic activation.

The present study allows the recognition of three modes of side-chain activation in enzymes. The first has been observed in a large number of enzymes in the absence of substrate. Active center or other functional groups often exhibit a chemical reactivity which is either unique or considerably enhanced, relative to the corresponding free amino acid (Vallee and Riordan, 1969). This atypical chemical behavior is quite characteristic of biologically active proteins and seems to be determined by the native protein environment.

The second mode has been observed consequent to substrate or substrate analog binding and is thought to reflect the adaption of the active-site topography to effective catalysis (Koshland and Neet, 1968). The slight increase observed in the rate of inactivation of aspartate aminotransferase by tetranitromethane on formation of noncovalent enzyme–substrate or –inhibitor complexes might result from such a mechanism (Figure 1, Table V).

The third, syncatalytic side-chain activation, as observed here with aspartate aminotransferase, only occurs in the course of the ensuing stages of catalysis, *i.e.*, during the bond-making and -breaking processes. As a consequence, in order to detect and delineate the phenomenon in yet other instances experimental conditions may have to be designed critically. No doubt, search for analogous syncatalytic chemical modifications with other enzymes may reveal additional examples.

The present results with aspartate aminotransferase provide direct experimental indication of a possible mechanism for multistep enzyme catalysis. The syncatalytic activation of a particular functional residue, produced by catalysis-linked conformational alterations of the enzyme–coenzyme–substrate complex, might well reflect the generation of a transient active-site topochemistry, optimally adapted for stabilization of an intermediate and/or catalysis of the neighboring steps in a multistep enzymatic reaction. Thus, the combination of conformational adaptability, syncatalytic changes in functional group reactivity, and multistage reaction might in certain instances be an essential feature of enzyme catalysis.

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Antibody to Adenosine Triphosphatase from Membranes of *Micrococcus lysodeikticus**

Theresa L. Whiteside† and Milton R. J. Salton

ABSTRACT: Antisera to Ca^{2+}-activated adenosine triphosphatase, isolated and purified from membranes of *Micrococcus lysodeikticus*, were prepared in rabbits and tested by the quantitative precipitin reaction. The antibody was specific for adenosine triphosphatase: it gave a single band against pure or crude enzyme and sonicated membranes; electrophoresis of the enzyme, followed by development with the antiserum gave a single band which had migrated toward the anode. The pooled antiserum contained approximately 0.4 mg of antienzyme/ml of serum. Complete inhibition of adenosine triphosphatase activity, noncompetitive with respect to the substrate, was obtained. Radial immunodiffusion was employed as a quantitative assay for adenosine triphosphatase.

The antibody to the *M. lysodeikticus* enzyme cross-reacted with and partially inhibited the activity of adenosine triphosphatases from membranes of *Sarcina flava*, *Sarcina lutea*, and *Micrococcus varians*. Treatment of the enzyme with 1% sodium dodecyl sulfate or 2.6 M guanidine hydrochloride yielded "subunits" which gave a precipitin line with antisera to membranes, but not with antiserum to adenosine triphosphatase.

Adenosine triphosphatase is a ubiquitous and important component of biological membranes of diverse origins including those of red blood cells (Askari and Rao, 1969), inner mitochondrial membranes (Racker, 1967), and the plasma membranes of bacterial cells (Abrams, 1965; Abrams and Baron, 1967, 1968; Evans, 1969). The enzyme has been identified as a particulate entity of the mitochondrial membrane (Racker and Horstman, 1967) and as an integral part of the red cell membrane (Marchesi and Palade, 1967). However, the identity of the "stalked" particles seen in negatively stained preparations of bacterial membranes (Abrams, 1965; Biryuzova *et al.*, 1964) has not yet been conclusively established, although there is clear-cut evidence that the ATPase[1] activity is associated with a well-defined particle possessing a central unit surrounded by six peripheral subunits (Muñoz *et al.*, 1968a). Furthermore, lead-staining techniques used in localization studies (Voelz and Ortigoza, 1968) yielded little information as to the precise mode of attachment or distribution of the enzyme on the bacterial membrane.

Muñoz *et al.* (1968b, 1969) purified a Ca^{2+}-dependent ATPase from the membranes of *Micrococcus lysodeikticus*, and it was identified as a major antigen of the membrane. As the enzyme could be readily purified, it became possible to prepare an antiserum specific for the membrane ATPase. It was our feeling that such an antiserum would provide a valuable reagent for structural studies as well as an aide in defining the properties of the enzyme itself. Moreover, such an antienzyme serum would be useful in recognizing structural and antigenic differences and similarities among ATPase proteins from different bacterial membranes. The present report deals with the preparation and characterization of a specific anti-ATPase serum.

Experimental Procedures

Purification of ATPase. Ca^{2+}-activated ATPase was obtained by a "selective release" method (Muñoz *et al.*, 1968b) from membranes of *M. lysodeikticus* (NCTC 2665) and purified by gel filtration on Sephadex G-200 as described by Muñoz

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[1] Abbreviations used in this work are: ATPase, adenosine triphosphatase or ATP phosphohydrolase (EC 3.6.1.3); Gd·HCl, guanidine hydrochloride.