Chemistry of the Inactivation of Cytosolic Aspartate Aminotransferase by Serine *O*-Sulfate†

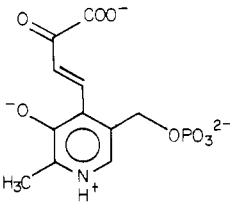
Hiroshi Ueno, John J. Likos,‡ and David E. Metzler*

ABSTRACT: The reaction of serine *O*-sulfate with cytosolic aspartate aminotransferase [John, R. A., & Fasella, P. (1969) *Biochemistry 8*, 4477] has been reinvestigated. As in the corresponding reaction with β-chloroalanine [Morino, Y., Osman, A. M., & Okamoto, M. (1974) *J. Biol. Chem. 249*, 6684], the enzyme is inactivated over a 10-min period, and the absorption maximum at pH 5.4 shifts from 430 to 336 nm. Upon prolonged standing the peak shifts again over a period of 20 h to 455 nm, a behavior entirely similar to that reported by Morino et al. for β-chloroalanine in the presence of 3 M formate. When the pH of either the 10-min product (**1a**) or the 20-h product (**1b**) is raised to 11 or above, a yellow, diffusible compound (**2**) is released from the protein. This compound as well as its dephosphorylation and reduction products has been isolated and studied by NMR spectroscopy. Compound **2** is identical with a compound formed from serine sulfate and glutamate decarboxylase by a similar reaction sequence [Likos, J. J., Ueno, H., Feldhaus, R. W., & Metzler, D. E. (1982) *Biochemistry* (preceding paper in this issue)] and is the product of an aldol condensation of pyruvate with pyridoxal phosphate. When the 20-h product **1b** is reduced with sodium borohydride and then heated in a boiling water bath, a material identical with the reduction product of **2** is released. We propose that the 20-h product **1b** consists of **2** bound to the enzyme. Pathways for the formation of the various compounds are proposed. These findings require a reevaluation of the mechanisms of action of many enzyme-activated inhibitors of pyridoxal phosphate dependent enzymes.

The cytosolic isoenzyme of aspartate aminotransferase from pig hearts is a dimer of identical 412-residue chains. The coenzyme pyridoxal phosphate is bound in Schiff base linkage to lysine-258. The three-dimensional structure is known (Arnone et al., 1981, 1982; Metzler et al., 1982). The general properties of the protein have been reviewed by Braunstein (1973). John & Fasella (1969) showed that serine *O*-sulfate causes irreversible inactivation of the enzyme and that the absorption band of the coenzyme at 430 nm at low pH or 362 nm at high pH is shifted to about 336 nm. Morino and associates (Morino et al., 1974, 1979; Morino & Tanase, 1978) studied the related reaction by which both cytosolic and mitochondrial isoenzymes are inactivated by β-chloroalanine. They showed that for the cytosolic enzyme the 336 nm absorption band is formed within 10 min. They also showed that during this period many molecules of pyruvate, Cl^-, and NH_4^+ are formed for every molecule of enzyme inactivated. When ^{14}C-labeled chloroalanine was used and if the 10-min product (**1a**) was reduced with sodium borohydride, over 85% of the radioactivity was retained with the protein following denaturation. A chymotryptic peptide carrying the radioactivity was isolated. From the sequence of this peptide it was deduced that the radioactive fragment was attached to the side chain of lysine-258. A plausible sequence of reactions to explain these results was the following: the inhibitory quasi-substrate forms a Schiff base, with the pyridoxal phosphate displacing Lys-258 by a "transimination" reaction. The α proton is removed as in a normal transamination reaction, after which the β-chlorine is eliminated as Cl^- to form a Schiff base of aminoacrylic acid (Scheme I). The side chain of Lys-258 was presumed to add to the double bond at the β carbon to give the inhibited enzyme. Because it absorbs at 336 nm, the product would logically be the ketimine shown in the scheme. Reduction wih borohydride would fix both the carbon atoms of the inhibitor and the coenzyme ring firmly to Lys-258.

A puzzling observation of Morino et al. was that in the presence of 3 M formate, the 10-min product was converted to a form (**1b**) absorbing at 455 nm over a period of 20 h. The structure of this form was unclear. It was also surprising that the peptides isolated from the modified active site possessed positive circular dichroism of unknown origin. The original absorption band of the coenzyme Schiff base at 362 nm at high pH completely disappeared when the 336-nm band was formed. It might be anticipated that the ketimine form of the inhibited enzyme would be in equilibrium with some isomeric aldimine form absorbing at 362 or 430 nm. Since both transamination and β elimination occur readily, even nonenzymatically, it is also strange that upon prolonged dialysis the chloroalanine-inactivated enzyme is not reactivated by conversion to the aldimine and elimination of lysine from the hypothetical inhibited enzyme. Nevertheless, the chemistry shown in the scheme has been accepted generally, and a similar sequence has been suggested for a variety of mechanism-based inhibitors acting on other aminotransferases (Morino et al., 1979; Soper & Manning, 1981; Fowler & John, 1981) and on other pyridoxal phosphate dependent enzymes [Seiler et al., 1978; Sandler, 1980; Bey, 1981; see also Likos et al. (1982b)].

One related reaction is the inhibition of glutamate decarboxylase of *Escherichia coli* by serine *O*-sulfate or β-chloroalanine. In both cases, an inactivated enzyme absorbing at 336 nm is formed. However, as shown by Likos et al. (1982b), a yellow product (**2**)[1] is released from serine sulfate



2

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‡ Present address: Monsanto Co., St. Louis, MO 63166.

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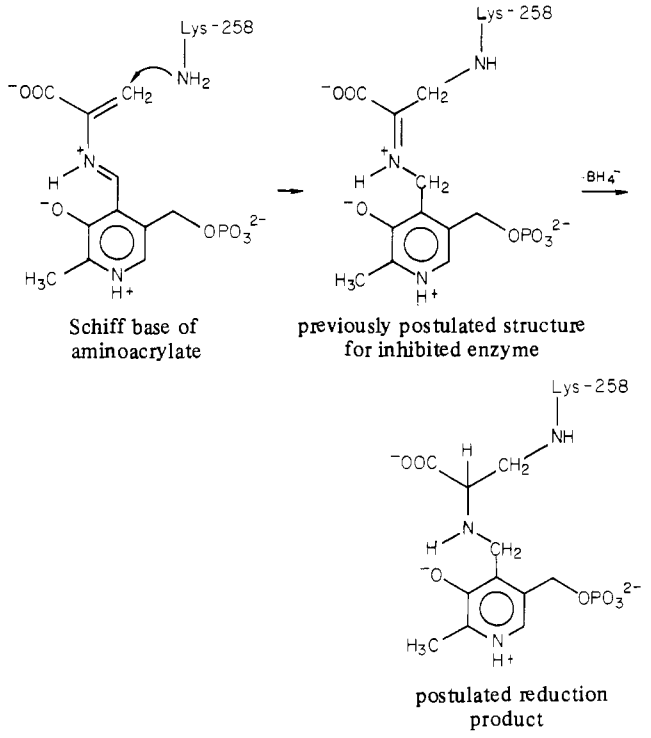
Scheme I



Schiff base of aminoacrylate

previously postulated structure for inhibited enzyme

postulated reduction product

inhibited glutamate decarboxylase by treatment with base. We show that the same product is released from aspartate aminotransferase inhibited with serine sulfate. We propose a sequence for its formation that differs significantly from that given in the preceding scheme. A preliminary report has been published (Likos et al., 1982a).

Materials and Methods

The reagents used and the equipment and procedures employed for UV-visible and NMR spectroscopy have been described in the preceding paper (Likos et al., 1982b).

Enzyme. The α subform of cytosolic aspartate aminotransferase was isolated from pig hearts as described by Martinez-Carrion et al. (1967). See also Yang & Metzler (1979).

Inactivation by Serine Sulfate (Preparation of Substances **1a–c**). In a typical reaction 25 mL of 2.8×10^{-4} M aspartate aminotransferase (316 mg of protein, 6.8 μmol of pyridoxal phosphate) in 0.04 M sodium acetate buffer, pH 5.4, was used. To this was added 70 mg of solid L-serine *O*-sulfate to give a concentration of 12.6 mM. After 10 min at room temperature, the enzyme was converted almost quantitatively to the inactive form **1a**. Over a period of 20 h at room temperature, the absorption maximum shifted from 336 to 455 nm (form **1b**). This form was stable as long as the solution was not dialyzed. However, dialysis against water for 8 h caused the disappearance of the 455-nm band and the formation of **1c**, a form characterized by a 430-nm absorption band at pH 5.4 and a 360-nm band at pH 8 (Morino et al., 1974).

Preparation of **2**. The pH of a solution of **1a**, **1b**, or **1c** was raised to 11.0 by the addition of 0.1 M KOH. The bright yellow **2** was separated from the protein by ultrafiltration through an Amicon PM10 membrane. When the volume of the protein solution had been reduced to 1–2 mL, it was diluted with 3–5 mL of water or of 0.01 M KOH and again ultrafiltered. The "washing" was repeated 2 more times. The combined filtrates contained over 80% of the original chromophore as judged by the absorbance at 420 nm.

The alkaline ultrafiltrate was passed through an 0.7 × 6 cm column of Dowex 50 (X8, 100–200 mesh) ion-exchange resin in the H^+ form. Very thoroughly washed resin was used. It was repeatedly treated alternately with 6 M NaOH and 6 M HCl. This cycle was repeated at least twice just prior to use, but the resin had been reused and rewashed many times before the crucial samples were prepared. Thorough washing was essential to avoid contamination of NMR samples in the aromatic region.

An alternative procedure made use of SP-Sephadex (SP-G25, 40–120-μm beads) in the H^+ form. This was prepared by treating the beads with 0.2 M HCl for 10 min and washing quickly with water. The treatment was repeated a second time. An 0.8 × 15 cm column was prepared and washed with about 6 L of water. Compound **2** from 100 mg of enzyme prepared with a minimum of KOH was passed through the column which was then washed with 20 mL of water. The combined effluent was lyophilized and taken up in D_2O for NMR spectroscopy. Compound **2** prepared in this way was free of aromatic impurities.

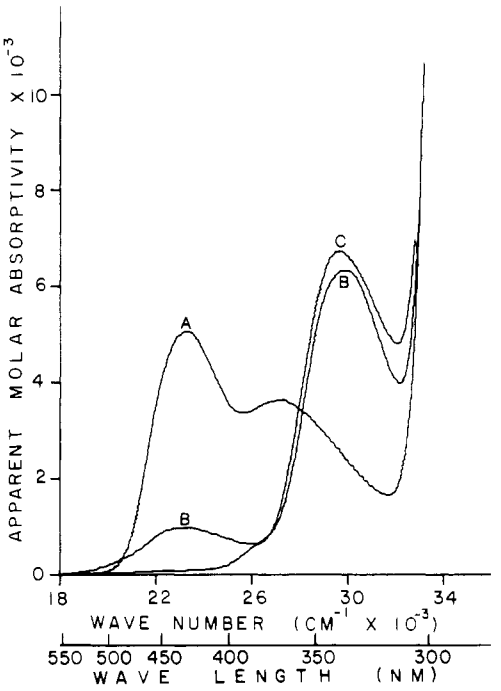


FIGURE 1: Reaction of cytosolic aspartate aminotransferase with L-serine *O*-sulfate. Solid serine sulfate (2 mg) was added to 400 μL of 2.5×10^{-4} M aspartate aminotransferase in 0.06 M sodium acetate buffer, pH 5.4 at room temperature. (A) Absorption spectrum of native enzyme. (B) Spectrum of inactivated enzyme (**1a**), 10 min after addition of serine sulfate. (C) Inactivated enzyme **1a** treated with sodium borohydride.

Results

In agreement with the report of John & Fasella (1969), treatment of aspartate aminotransferase with L-serine *O*-sulfate causes a rapid loss of the 430-nm absorption band present at low pH, an appearance of a new band at 336 nm (Figure 1), and inactivation of the enzyme. With 10 mM serine sulfate, over 95% of the catalytic activity is lost within 5 min at 25 °C. We designate the product of this initial reaction **1a**.

[1] Abbreviations: compound **2**, 4-[3-hydroxy-2-methyl-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxo-3-butenoic acid; compound **3**, 4-[3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]-2-oxo-3-butenoic acid; compound **4**, 4-[3-hydroxy-2-methyl-5-[(phosphooxy)methyl]-4-pyridinyl]-2-oxobutyric acid; compound **5**, 3-(1,3-dihydro-7-hydroxy-6-methyl-1-furo[3,4-*c*]pyridinyl)-2-oxopropionic acid; compound **6**, 4-[3-hydroxy-5-(hydroxymethyl)-2-methyl-4-pyridinyl]butyric acid; Tris, tris(hydroxymethyl)aminomethane.

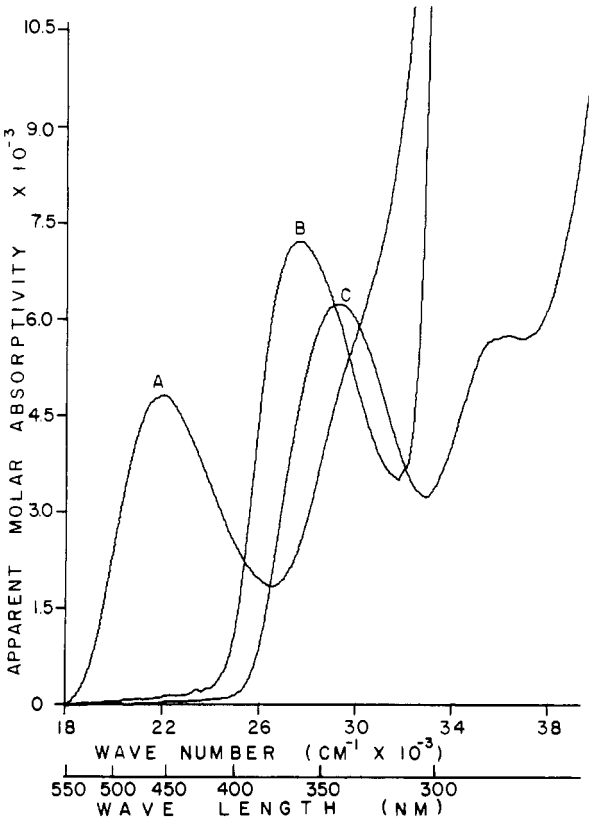


FIGURE 2: Formation and reactions of the second product (**1b**) formed from the action of serine *O*-sulfate on aspartate aminotransferase. (A) Spectrum of **1b** formed by allowing inactivated enzyme **1a** (Figure 1) to stand for 20 h at room temperature at pH 5.4. (B) Product of reduction of **1b** with sodium borohydride. (C) Spectrum of the supernatant of reduced **1b** after dialysis against water followed by heat denaturation (2-min immersion in a boiling water bath). This product is compound **4** (see text).

Over a 20-h period at room temperature, **1a** is converted to a second inactive species, **1b**, which has an absorption maximum at 455 nm (Figure 2). This conversion occurs at either pH 5.4 or pH 8 and under either O_2 or N_2 at similar rates. However, at 5 °C form **1a** is quite stable and is transformed only very slowly into **1b**. It should be noted that conversion of **1a** to **1b** never seems to be quite complete, a shoulder of variable height remaining at about 336 nm.

As observed previously by Morino et al. (1974), the spectrum of **1b** is altered by dialysis against water, the absorption maximum shifting from 455 to 360 nm (**1c**; Figure 3). The process is reversible, dialysis against 3 M formate converting the 360-nm band (**1c**) back to 455 nm. The 455-nm band is formed by reaction of the enzyme with serine sulfate at either pH 8.3 or pH 5.4. No change in spectrum with pH is seen. However, after dialysis against water, the 360-nm band of **1c** shifts reversibly to 430 nm when the pH is lowered to 5.4.

Products **1a**, **1b**, and **1c** are similar in that treatment with NaOH to raise the pH to about 11 leads to nearly quantitative release of the coenzyme as yellow compound **2** whose structure has been established (Likos et al., 1982b) and which has been synthesized previously by Schnackerz et al. (1979). Compound **2** can be converted to compounds **3**–**6** by dephosphorylation and reduction (Scheme II). These compounds have been prepared both from **2** isolated from aspartate aminotransferase and from synthetic **2**, and their chemical properties have been compared. The positions of the absorption maxima at neutral pH are given in Scheme II. The yields and apparent molar absorptivities of compounds **2**, **3**, and **5** are given by Ueno (1982).

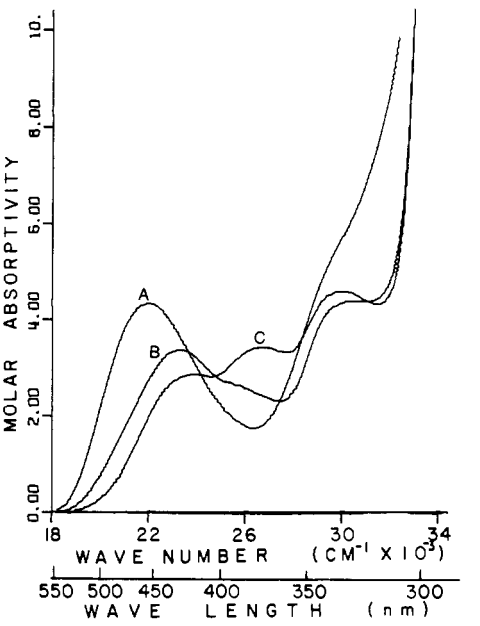
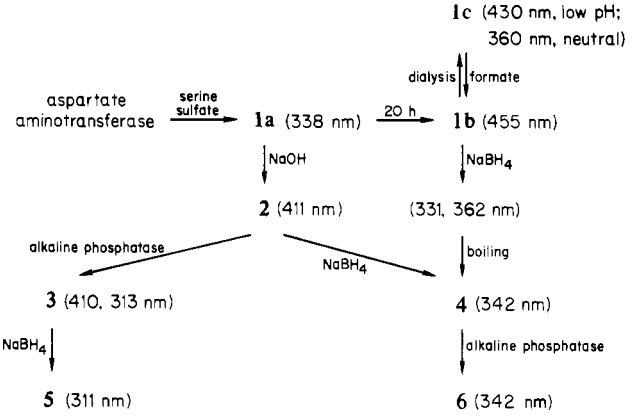


FIGURE 3: Formation and reaction of the third product (**1c**) formed from the action of serine *O*-sulfate on aspartate aminotransferase. (A) Absorption spectrum of **1b** at pH 5.4. Product **1c** was formed by dialysis of **1b** against a buffer for 24 h. (B) Spectrum of **1c** at pH 5.4 acetate buffer. (C) Spectrum of **1c** at pH 8.4 Tris buffer.

Scheme II



The proton NMR spectrum of **2** isolated from aspartate aminotransferase is shown in the preceding paper (Likos et al., 1982b) where it is compared with that of synthetic **2**. Its identity is established conclusively by this result. The proton NMR spectra and ultraviolet spectra of derivatives **3**, **4**, **5**, and **6** obtained from **2** isolated from aspartate aminotransferase are also identical with those for the corresponding derivatives from synthetic **2** and **3**.

Properties of **1a**. The absorption band of **1a** at 336 nm is in the same position as that of the corresponding inactivated form of bacterial glutamate decarboxylate (Likos et al., 1982b). Compound **1a** is stable at low temperature and against prolonged (1 week) dialysis against water at 4 °C. Treatment of **1a** with solid sodium borohydride causes no change in the spectrum (Figure 1). Incubation of either **1a** or reduced **1a** with 0.3 mM pyridoxal phosphate did not cause any reactivation of the enzyme.

Heating **1a** in a boiling water bath for 2 min denatures the protein and releases about 50% of the chromophore in a form (presumably **2**) absorbing at 403 nm at pH 5.4. The same treatment of reduced **1a** releases no chromophore, nor does treatment with sodium hydroxide release **2**. This is in agreement with the observation of Morino et al. (1974) that

Table I: Chemical Shifts[a] from ^1H NMR Spectrum of Compound 4

	deuterated[b] 4 at pD 1		undeuterated compound at pD 2.2		pD 6.9		pD 12.9 (50 °C)	
protons	ppm	Hz	ppm	Hz	ppm	Hz	ppm	Hz
2'-CH_3	2.67		2.66		2.45		2.21	
4'-H	6.86	J = 16.1	6.84	J = 17.6	6.73	J = 16.1	6.70	J = 15.1
	6.91		6.89		6.83		6.75	
5'-CH_2	5.03	J = 7.3	5.01	J = 7.8	4.89	J = 6.3	4.67	J = 4.4
	5.05		5.04		4.91		4.68	
6-H	8.25		8.23		7.73		7.53	
α-H			5.12	J = 5.4			4.57	J = 6.8
			5.13				4.60	
β-H	6.68 }	J = 16.6	6.67	J = 5.4	6.67	J = 5.4	6.30	J = 6.8
			6.68		6.69		6.33	
	6.74 }		6.72	J = 4.9	6.72	J = 5.4	6.36	J = 6.8
			6.74		6.74		6.38	

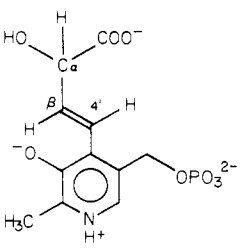
[a] At 25 °C. Chemical shifts in ppm relative to Me_4Si as an external standard. [b] One atom of ^2H incorporated at C_α by reduction of **2** with sodium borodeuteride.

reduction fixes the chromophore to the protein. Heat denaturation of the native enzyme under the same conditions releases about 50% of the pyridoxal phosphate. Again, as is well-known, reduction fixes the coenzyme to the protein.

Properties of **1b**. The absorption spectrum of **1b** has a maximum at 455 nm whose position and intensity are constant from pH 5.4 to 8.3. It is devoid of circular dichroism. Dialysis against water or against 0.1 M acetate buffer, pH 5.4, or 0.1 M triethanolamine hydrochloride buffer, pH 8.3, with or without addition of 0.3 mM pyridoxal phosphate causes no reactivation of the enzyme. Form **1b** is very stable in 0.06 M acetate buffer at pH 5.4 and rapidly forms large orange crystals in the presence of 8% poly(ethylene glycol) of molecular weight 4000 (Metzler et al., 1979). However, the resulting crystals are not isomorphous with those of the native enzyme (A. Arnone, personal communication).

Dialysis against either 0.1 M acetate buffer or triethanolamine buffer converts **1b** to **1c**. However, in agreement with Morino et al. (1974), no change in spectrum is seen when **1b** is dialyzed against 3 M sodium formate. Heating of **1b** in a boiling water bath releases only about 10% of the bound chromophore. However, when **1b** is heated in 3 M formate at pH 5.4, the chromophore is released quantitatively. The released material has an absorption maximum at 410 nm at pH 5.4, and its UV spectrum at other pH values suggests that it is **2**. Reduction of this released material with sodium borohydride gives a product with properties appropriate for **4**.

Treatment of **1b** with either sodium borohydride or sodium cyanoborohydride shifts the absorption maximum from 455 to 361 nm (Figure 2). As reported by Morino et al. (1974), treatment with 6 M guanidine hydrochloride denatures reduced **1b** and releases about 80% of the chromophore as a low molecular weight material (Figure 3). This material, which we identify as compound **4**, is obtained in purer form if reduced



4

1b is thoroughly dialyzed against water and is then heated in a boiling water bath. As is clear from Figure 4 and Table I,

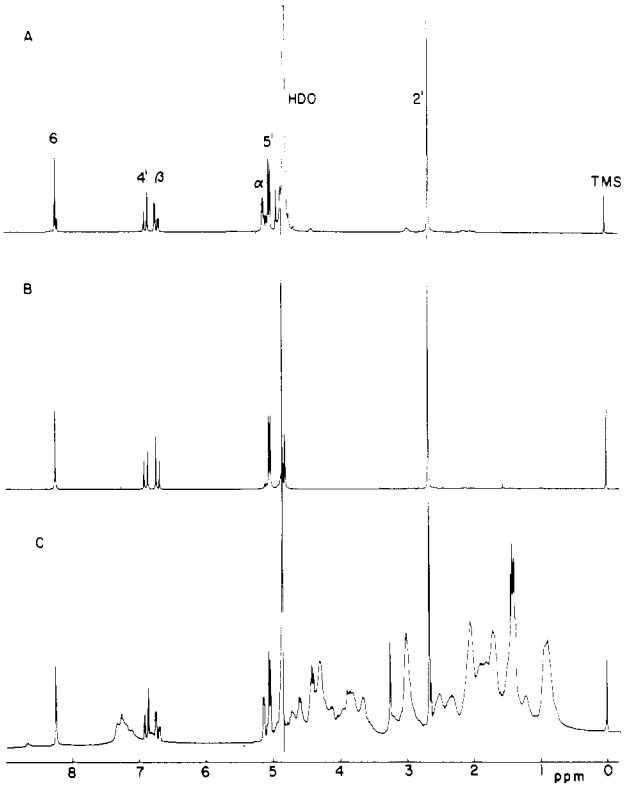


FIGURE 4: Proton NMR spectra of **4** obtained on a 300-MHz spectrometer at 24 °C. (A) Synthetic **4** (~10 μmol) at pD ~2.2. (B) Synthetic **4** with one deuterium atom incorporated in the α position (~16 μmol), pD ~1. (C) **4** isolated from **1b** after its reduction with sodium borohydride and heat denaturation. The release **4** was acidified with a drop of 6 N HCl and was then lyophilized several times in D_2O. The chemical shifts are relative to an external Me_4Si standard at 0 ppm.

the released compound has the same NMR spectrum as **4**. Of special importance is the pair of coupled resonances with J = 16.1 Hz representing the 4' and β protons of **4**. The proton giving rise to the upfield (6.71-ppm) doublet is coupled further with J = 5.4 Hz to a single proton at δ 5.13. The latter proton must be the α-H while the β-H gives the 6.7-ppm doublet. The spectrum of compound **4** prepared by reduction of **2** with sodium borodeuteride lacks the α-proton peak and the associated coupling (Figure 4).

We could not completely rule out the possibility that the released product was an amino acid rather than the alcohol **4**. We therefore compared it with **4** by high-voltage electro-

phoresis at pH 6.5. The two compounds migrated identically when side by side or when mixed as a single spot. Ultraviolet–visible absorption spectra are also identical. We conclude that the unknown is **4**.

The treatment of **1b** with 9 mM β-mercaptoethanol or with 16 mM cysteine sulfinate slowly (overnight) converted it to products similar to those obtained by borohydride reduction with an absorption maximum at 360 nm and a negative circular dichroism band at about 365 nm. The conversion with β-mercaptoethanol was only about 50% complete.

Attempts were made to remove the chromophore from **1b** by displacement with phosphate by using the procedure employed for resolving the native enzyme of pyridoxamine phosphate (Scardi et al., 1963; Yang & Metzler, 1979). Warming at 37 °C with 1 M sodium phosphate at pH 5 did not alter the UV spectrum of **1b** during a 24-h period.

Properties of Form **1c**. The spectrum of the dialyzed form **1c** resembles that of the native enzyme, the absorption band shifting partially from 430 nm at low pH to 360 nm at high pH (Figure 3). However, it is catalytically inactive and forms **2** when treated with NaOH. Heating **1c** in a boiling water bath also appears to produce **2** quantitatively.

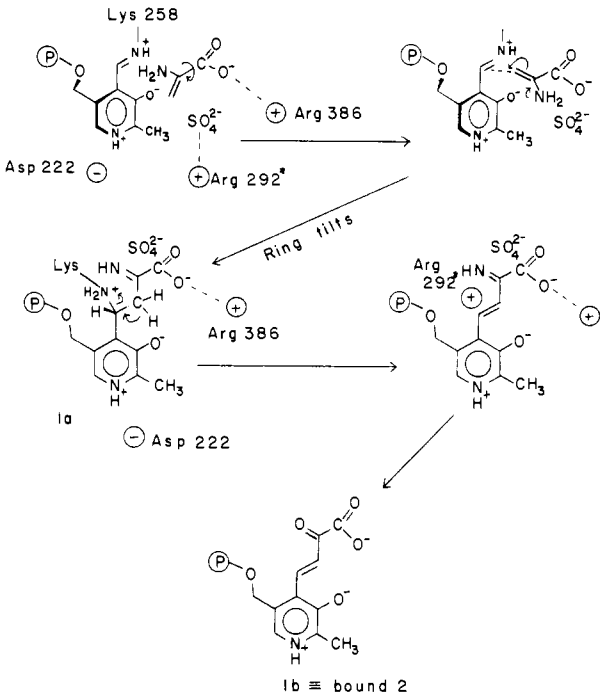
Form **1c** is converted back to **1b** by the addition of ammonium formate at a concentration of 0.14 M or less. Reduction of **1c** with sodium borohydride gives a band absorbing at 360 nm, just as does reduction of **1b**. Upon heat denaturation, compound **4** is apparently released.

Discussion

A comparison of NMR spectra, UV–visible absorption spectra, and electrophoretic mobilities of synthetic keto acid **2** with **2** isolated from aspartate aminotransferase leaves no doubt as to their identity. In addition, the dephosphorylated and reduced derivatives **3**–**6** all are identical when prepared from synthetic or isolated **2**.

The formation of **2** when serine sulfate inhibited aspartate aminotransferase is treated with base is strongly indicative of the formation of free aminoacrylate by the enzyme and of attack by the latter upon the 4′-carbon atom of the Schiff base. However, since a molecule of enzyme produces about 100 molecules of pyruvate and ammonia before it is inactivated (John & Fasella, 1969), it is clear that most of the aminoacrylate formed is released and does not react with the Schiff base. Scheme III shows various groups in approximately the positions they occupy in the known three-dimensional structure of the crystalline enzyme (Arnone et al., 1982). The first structural formula shows aminoacrylate as it presumably sits in the active site after being released from its Schiff base with pyridoxal phosphate by transimination. The carboxyl group of the aminoacrylate is bound by arginine-386, and the eliminated sulfate presumably occupies the site of its initial binding on arginine-292 of the other subunit (Arg-292*). It can be seen that by a simple 180° rotation about the carbon–carbon bond to its carboxyl the aminoacrylate could assume a conformation in which its β carbon is ready for nucleophilic attack on carbon 4′ of the coenzyme. We propose that the resulting adduct is product **1a**. It is likely that the coenzyme tilts forward toward the substrate during the addition as it does during the transimination of normal substrates (Karpeisky & Ivanov, 1966; Ivanov & Karpeisky, 1969; Jansonius et al., 1981; Arnone et al., 1982). The structure of **1a** is drawn looking up from below directly at the face of the ring after it has tilted forward. One uncertainty is whether or not **1** contains the C=NH group pictured or whether this has been hydrolyzed to C=O with loss of ammonia. Peptide work in progress should answer this question.

Scheme III



Compound **1a** has a chiral center at the 4′-carbon atom, a fact that doubtless explains the positive circular dichroism observed by Morino & Okamoto (1973) and by Morino & Tanase (1978) for peptides isolated from both chloroalanine-inactivated cytosolic and mitochondrial aspartate aminotransferase. The shortest chymotryptic fragments, which were tetrapeptides, retained the circular dichroism observed for the intact inactivated enzyme. It is of interest that the circular dichroism of chloroalanine-inactivated alanine aminotransferase is negative (Morino et al., 1979), a fact that may indicate the opposite chirality of the derivative analogous to **1** formed with that enzyme.

Of special interest for aspartate aminotransferase is the slow formation of product **1b** which absorbs at 455 nm. This appears to be identical with the product formed from β-chloroalanine in the presence of 3.5 M formate (Morino et al., 1974) and may be closely related to a product formed slowly by the action of β-chloroalanine on alanine aminotansferase (Golichowski & Jenkins, 1978). When **1b** is treated with sodium borohydride the resulting reduced coenzyme derivative is released by heating as compound **4**. On this basis we propose that **1b** is the keto acid **2** bound at the active site. The imine group that is pictured in this scheme as present in **1a** must be lost by hydrolysis either prior to or immediately after elimination of the lysine-258 side chain from **1a**.

When **2** was incubated with apo-aspartate aminotransferase, **1b**, was not formed, but a complex spectrum with overlapping lower wavelength bands was produced. However, Schnackerz et al. (1979) showed that **2** binds slowly to apo-D-serine dehydratase to give a band at 460 nm, very similar to that of **1b**.

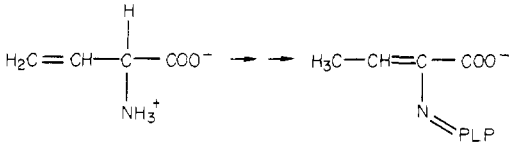
The nature of form **1c** is not clear. Borohydride reduction does not fix the coenzyme to the protein. Therefore, it is unlikely that it is a Schiff base of **2** with Lys-258. It seems to us possible that the sulfate eliminated from the inhibitor remains bound in the active site in **1b** or is replaced by formate. Form **1c** may have lost this sulfate and have undergone a change in protein conformation which allows the observed pH-dependent change in spectrum. It seems reasonable that the 430-nm band at low pH could be caused by **2** if the double

bond containing side chain were rotated out of coplanarity with the ring. It is hard to see how a simple deprotonation could shift the band to a shorter wavelength at high pH. Another possibility is that at high pH some nucleophile adds to the carbonyl group of the bound **2**. This could by Lys-258 forming a carbinolamine rather than a Schiff base.

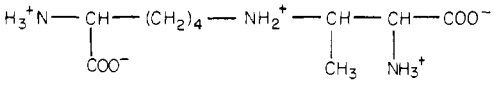
Using uniformly ^{14}C-labeled serine sulfate, John et al. (1973) showed that a labeled protein was obtained by denaturation and carboxymethylation of **1a** followed by digestion with thermolysin. The peptide had a 330-nm absorption band, and amino acid analysis gave phenylalanine, serine, asparagine, glycine, and what was thought to be (carboxymethyl)cysteine. The N-terminal sequence was Phe-Ser. There is no cysteine-containing sequences that could give rise to such a peptide (Ovchinnikov et al., 1971). We suspect that this peptide actually contained the active-site sequence Phe-Ser-Lys*-Asn-Phe-Gly where Lys* is the modified lysine expected from degradation of our proposed structure of **1a**. Experiments are in progress to test this possibility.

Both cytosolic aspartate aminotransferase and bacterial glutamate decarboxylase are evidently inactivated in a similar manner by aminoacrylate generated in the active site. A question of obvious interest is to what extent similar mechanisms apply to suicide inactivators acting on other enzymes [see Seiler et al. (1978); Sandler, 1980; Bey, 1981]. An important example is γ-aminobutyrate aminotransferase of brain which is inhibited by ethanolamine *O*-sulfate (John & Fowler, 1976; Fowler & John, 1981). In this instance the inactivated enzyme has an absorption band at 330 nm, but the 415-nm band of the native enzyme is only partially gone. This suggests that the chemistry of Scheme I may be correct for this enzyme. The ketimine product could be in equilibrium with some aldimine. On the other hand, the 415-nm band of the inhibited enzyme could resemble product **1b** of the present study. γ-Aminobutyrate aminotransferase is also inhibited by 4-amino-5-halopentenoic acids (Silverman & Levy, 1981). The changes in absorption spectrum are similar to those observed with ethanolamine *O*-sulfate.

A larger number of suicide inactivators of pyridoxal phosphate dependent enzymes contain carbon–carbon double bonds in such a position that isomerization can lead to formation of a Schiff base of pyridoxal phosphate (PLP) with α,β unsaturation. An example is vinylglycine:



Again a 335-nm absorption band appears upon reaction with aspartate aminotransferase (Rando, 1974). Degradation of the inhibited enzyme gave a chymotryptic tetrapeptide from the active site which was converted by acid hydrolysis to a derivative of lysine-258 that cochromatographed with homoserine lactone (Gehring et al., 1977). Its proposed structure is



This amino acid presumably arose from addition of the amino group of Lys-258 to the β carbon of the α,β-unsaturated Schiff base. The 335-nm band was assigned to an adduct between the resulting β-substituted Schiff base and another nucleophile from the protein. An unusual stability toward removal of the coenzyme was noted. This suggests the possibility of an alternative chemistry related to that described here. Pyridoxal phosphate was reported to be released by treatment with either NaOH or trichloroacetic acid. This would seem contrary to our suggestions. However, our compound **2** is very unstable in strong base and is converted to pyridoxal phosphate. Therefore, it is still possible that the 335-nm form is an adduct analogous to our **1a**. A more specific inhibitor of aspartate aminotransferase is β-methyleneaspartic acid (Cooper et al., 1982). The chemistry of its action is presumably analogous to that of vinylglycine.

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Nitrogenase Reactivity: Cyanide as Substrate and Inhibitor†

Jia-ge Li,‡ Barbara K. Burgess,* and James L. Corbin

ABSTRACT: We have examined the reduction of cyanide by using the purified component proteins of nitrogenase (*Av*1 and *Av*2). The previously reported self-inhibition phenomenon was found to be an artifact. One of the two species present in cyanide solutions, CN^-, was shown to be a potent reversible inhibitor (K_i = 27 μM) of *total* electron flow, apparently uncoupling MgATP hydrolysis and electron transfer. There appears to be no differential effect of CN^- on the specific activities of *Av*1 and *Av*2 nor is there any apparent irreversible physical damage to *Av*2. CN^- inhibition is completely reversed by low levels of CO, implying a common binding site. Azide partially relieves the inhibitory effect, but other substrates and inhibitors (N_2, C_2H_2, N_2O, H_2) have no effect. The other species present in cyanide solutions, HCN, was shown to be the substrate (K_m = 4.5 mM at *Av*2/*Av*1 = 8), and extrapolation of the data indicates that at high enough HCN concentration H_2 evolution can be eliminated. The products are methane plus ammonia (six electrons), and methylamine (four electrons). There is an excess (relative to methane) of ammonia formed, which, according to electron balance studies, may arise from a two-electron intermediate. Both nitrous oxide and acetylene (but not N_2) influence the distribution of cyanide reduction products, implying simultaneous binding. HCN appears to bind to and be reduced at an enzyme state more oxidized than the one responsible for either H_2 evolution or N_2 reduction.

Nitrogen fixation and all other reductions catalyzed by the nitrogenase system require two easily separated component proteins, called the molybdenum–iron protein (MoFe protein) and the iron protein (Fe protein). The physical properties of these two proteins have been recently reviewed (Orme-Johnson et al., 1977; Mortenson & Thorneley, 1979), and great similarity among proteins from different bacterial sources is evident (Emerich & Burris, 1976a,b). In addition to these two proteins, a source of reducing equivalents, MgATP, and protons are required for all substrate reductions (Bulen & LeComte, 1966). The MoFe protein is believed to contain the site of substrate reduction (Shah et al., 1973; Hageman & Burris, 1979), while the Fe protein is generally accepted as the specific one-electron donor for the MoFe protein (Hageman & Burris, 1978a,b; Ljones & Burris, 1978a,b). In addition to N_2, nitrogenase catalyzes the reduction of protons, nitrous oxide, acetylene, azide, cyanide, alkyl cyanides, alkyl isocyanides, hydrazine, cyclopropene, and allene. Alternative substrates have often been studied as probes for the number and nature of sites of substrate interaction on nitrogenase and the types of intermediates that might be formed during N_2 reduction. This paper will describe recent studies on the reduction of cyanide by the purified component proteins of nitrogenase.

Cyanide reduction by nitrogenase was first demonstrated by Hardy & Knight (1967). Using crude nitrogenase preparations, they showed that cyanide was reduced by six electrons to methane plus ammonia with small amounts (10% of the NH_3) of another base which was suggested to be a four-electron product, methylamine. The formation of all products required both component proteins, MgATP, and a reductant, and all were completely inhibited by carbon monoxide (0.9 atm). These observations were subsequently confirmed by a number of other investigators (Hwang & Burris, 1972; Rivera-Ortiz & Burris, 1975; Kelly et al., 1967; Kelly, 1968). Hardy & Knight (1967) suggested that cyanide reduction proceeded via the two-electron intermediates, methylenimine and methylamine, and was a good model for N_2 reduction. They were unable, however, to demonstrate the reduction of methylamine to methane and ammonia. Kelly et al. (1967) observed very small amounts of ethylene and ethane (0.08% of CH_4) during cyanide reduction catalyzed by nitrogenase. They suggested that these C_2 products were formed by interaction of adjacent C_1 radicals on adjacent cyanide binding sites.

Several investigators (Hardy & Knight, 1967; Hwang & Burris, 1972; Rivera-Ortiz & Burris, 1975) reported that the rate of nitrogenase-catalyzed cyanide reduction appeared to

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‡ Present address: Institute of Botany, Peking, People's Republic of China.

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