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Inhibition of Alanine Racemase by Alanine Phosphonate: Detection of an Imine Linkage to Pyridoxal 5'-Phosphate in the Enzyme-Inhibitor Complex by Solid-State ^{15}N Nuclear Magnetic Resonance†

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ABSTRACT: Inhibition of alanine racemase from the Gram-positive bacterium *Bacillus stearothermophilus* by (1-aminoethyl)phosphonic acid (Ala-P) proceeds via a two-step reaction pathway in which reactivation occurs very slowly. In order to determine the *mechanism* of inhibition, we have recorded low-temperature, solid-state ^{15}N NMR spectra from microcrystals of the [^{15}N]Ala-P–enzyme complex, together with spectra of a series of model compounds that provide the requisite database for the interpretation of the ^{15}N chemical shifts. Proton-decoupled spectra of the microcrystals exhibit a line at ~150 ppm, which conclusively demonstrates the presence of a protonated Ala-P–PLP aldimine and thus clarifies the structure of the enzyme–inhibitor complex. We also report the pH dependence of Ala-P binding to alanine racemase.

The alanine racemases are a group of pyridoxal 5'-phosphate (PLP)[1] containing bacterial enzymes that catalyze the racemization of L- and D-alanine. These enzymes are operative at an early stage of cell growth, thus providing D-alanine for inclusion into the peptidoglycan component of the bacterial cell wall. Since the integrity of the peptidoglycan layer is crucial to bacterial growth, the racemases have been a target for antibacterial drug development, and thus the mechanism of inhibition of these enzymes is of considerable interest.

The inactivation of alanine racemase from Gram-positive *Streptococcus faecalis* and *Bacillus stearothermophilus* by the alanine analogue (1-aminoethyl)phosphonic acid (Ala-P) exhibits an unusual kinetic behavior. Specifically, Gram-positive racemases show time-dependent inhibition by Ala-P, the enzyme–Ala-P complex exhibiting slow dissociation (Badet & Walsh, 1985; Badet et al., 1986; Atherton et al., 1979; Allen et al., 1979). The proposed mechanism of inhibition of *B. stearothermophilus* alanine racemase by Ala-P is shown in Figure 1. Ala-P initially binds in a weak, reversible fashion (K_i = 1 mM), followed by isomerization to yield the enzyme–Ala-P complex that subsequently dissociates with $t_{1/2}$ = 25 days (Badet et al., 1986). Although the kinetics of inactivation have been determined, the structure of the long-lived, inactive complex is unknown. Normal catalytic turnover of alanine proceeds by initial transaldimination of the resting enzyme to form the PLP–substrate aldimine, followed by abstraction of the α-proton (Faraci & Walsh, 1987). Ala-P, an α-aminophosphonic acid, should also be capable of transaldimination to form the corresponding Ala-P–PLP aldimine, and the fluorescence and CD spectra of the active and inactive (Ala-P) enzyme species are consistent with the occurrence of the process (Badet et al., 1986). However, unlike the PLP–substrate aldimine, the inactive enzyme species EI* is not detectably reduced by borohydride, suggesting that perhaps the Ala-P–enzyme complex may not form an imine.[2]

One method which may be used to resolve these conflicting experimental results is ^{15}N NMR. It has been known for some time that ^{15}N chemical shifts are extremely sensitive to covalent bonding characteristics and to protonation/deprotonation of nitrogens. For example, the ^{15}N resonance of $-^{15}NH_3^+$, such as is present in Ala-P, occurs at ~0 ppm (relative to external NH_4Cl), whereas lines due to protonated imines are shifted to ~150 ppm. Thus, it is quite easy to distinguish between free Ala-P and an Ala-P–PLP protonated Schiff base on the basis of their chemical shifts (Allen & Roberts, 1980; Botto & Roberts, 1979; Harbison et al., 1983). Finally, if the Ala-P–PLP Schiff base is deprotonated, an additional downfield shift to ~300 ppm occurs (vide infra).

The molecular weight of alanine racemase (a homodimer of M_r 78 000) is quite large by the standards of high-resolution, solution NMR. In particular, the size of the protein leads to long correlation times and therefore to broad NMR lines. In addition, a variety of other problems are often encountered in systems of this size, including aggregation and/or precipitation at concentrations necessary for NMR, and in this particular case, exchange of the NH proton occurs, which

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[1] Abbreviations: Ala-P, (1-aminoethyl)phosphonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CP, cross polarization; CW, continuous wave; DAAO, D-amino acid oxidase; LDH, L-lactate dehydrogenase; MAS, magic angle spinning; NADH, nicotinamide adenine dinucleotide (reduced form); NMR, nuclear magnetic resonance; PEG, poly(ethylene glycol); PLP, pyridoxal 5'-phosphate; PNP, pyridoxamine phosphate; ppm, parts per million.

[2] Borohydride has been shown to add to the resting enzyme lysyl aldimine to yield an inactive PNP-enzyme species.

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$$E + I \underset{K_i}{\rightleftharpoons} E{\cdot}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E{\cdot}I^*$$

FIGURE 1: Reaction scheme for *B. stearothermophilus* alanine racemase in activation by Ala-P. The first step occurs reversibly with K_i = 1 mM as shown, followed by formation of the inactive complex with k_2 = 10 min^{-1} and k_{-2} = 5 × 10^{-5} min^{-1} (Badet et al., 1986).

makes polarization transfer or indirect detection of ^{15}N resonances difficult. These latter techniques have been mandatory to overcome the low sensitivity inherent in ^{15}N protein solution spectra (Bax et al., 1983; Griffey et al., 1985; Griffey & Redfield, 1987; McIntosh et al., 1987). We have chosen to circumvent these problems by employing low-temperature, high-resolution, solid-state NMR methods in our studies of the inhibition of alanine racemase. In this case we have performed MAS NMR experiments on microcrystals of the Ala-P–enzyme complex prepared by using ^{15}N-labeled Ala-P inhibitor. The low temperatures provide enhanced signal-to-noise for the NMR experiment, in addition to long-term stability for the enzyme–inhibitor complex. Under these circumstances, ^1H-decoupled ^{15}N MAS spectra exhibit centerbands at the isotropic (solution) resonance, flanked by sidebands spaced at the spinning frequency. The ratios of the sideband and centerband intensities permit a determination of the shift anisotropies, and these data, as well as the isotropic shifts, are employed in establishing the structure of the inactive enzyme complex. For example, protonated Schiff bases exhibit chemical shift tensors of about 300 ppm breadth, whereas deprotonation leads to a tensor of about twice that size (Harbison et al., 1983).

Herein we present evidence for the structural identification of the inactive Ala-P–enzyme complex by ^{15}N MAS NMR. Specifically, we observe a chemical shift and shift anisotropy consistent with formation of an Ala-P–PLP complex containing a protonated Schiff base linkage. To our knowledge, this is the first time that solid-state NMR has been employed to elucidate structural features of an unknown enzyme–inhibitor complex. Our success demonstrates that this approach should be generally applicable to large proteins (M_r ~100 000) and should also provide a means to trap enzyme–substrate and unstable enzyme–inhibitor complexes at low temperatures and to examine their NMR spectra.

Experimental Procedures

Materials. L-Alanine, NADH, α-ketoglutarate, and CHES were purchased from Sigma Chemical Co. Lactate dehydrogenase (10 mg/mL; 550 units/mg) from porcine muscle and D-amino acid oxidase (5 mg/mL; 15 units/mg) from porcine kidney were obtained from Boehringer-Mannheim Biochemicals. L- and D-Ala-P were purchased from Fluka Chemical Co.

Alanine racemase from Gram-positive *B. stearothermophilus* was prepared from the clone of gene in an *Escherichia coli* overproducing strain according to the method of Neidhart et al. (1987). The enzyme was purified to >95% homogeneity in ca. 100-mg quantities as determined by polyacrylamide gel electrophoresis.

Synthesis of [^{15}N]Ala-P. To 1.0 g of [^{15}N]benzamide (8.2 mmol), purchased from ICN Biomedicals, Inc., 99% pure, in 10 mL of 1,2-dichloroethane was added 2.5 mL of triphenyl phosphite (9.2 mmol), and the mixture was stirred. A total of 20 μL of boron trifluoride (40% in acetic acid) was added and the solution heated to 60 °C; then 0.8 mL of acetaldehyde (15 mmol) was added and the solution heated at 75 °C for 1 h. The solution was subsequently cooled to room temperature and allowed to stir overnight. Dichloroethane was evaporated under reduced pressure, 20 mL of 6 N HCl and 4 mL of glacial acetic acid were added, and the solution was refluxed for 5 h. Phenol and benzoic acid were removed by extraction with methylene chloride, and the water layer was evaporated under reduced pressure. Excess acid was removed by repeated addition and removal (by evaporation) of water. [^{15}N]Ala-P was isolated by crystallization from an ethanol/H_2O mixture. ^1H NMR chemical shifts [^1H (^2H_2O) δ 1.3 (m, 3, $-CH_3$), 3.15 (m, 1, $-CH$)] confirmed the presence and purity of Ala-P.

Synthesis of [^{15}N]Ala-P–PLP Schiff Base. A total of 0.5 g of PLP (2 mmol) was dissolved in 50 mL of H_2O to which 0.31 g of [^{15}N]Ala-P (2 mmol) was added, and the pH was adjusted to either 7.5 or 13.0 with NaOH. Absorption spectra showed a λ_{max} at 414 nm (at pH 7.51) and λ_{max} at 365 nm (at pH 13.0) indicative of protonated and unprotonated Schiff bases, respectively (Kallen et al., 1984; Langohr & Martell, 1978). The solutions were lyophilized and 0.8 g of solid was isolated.

Analytical Methods. Enzyme inactivation experiments with Ala-P were performed at 37 °C in 0.1 M CHES buffer at pH 9.1. Absorption spectra and steady-state reaction rates were measured with a Perkin-Elmer 554 spectrophotometer.

Inactivation Kinetics. Competitive inhibition was ascertained by measuring the Michaelis–Menten parameters of L-alanine to D-alanine racemization at various L-Ala-P concentrations. To 0.9 mL of CHES buffer were added 20 μL of DAAO, 5 μL of LDH, 0.2 mM NADH, and 10 μL of L-Ala-P and L-alanine varying from 8 to 40 mM. Racemase was added and the steady-state values were calculated in the presence of inhibitor (L-Ala-P) concentrations ranging from 0 to 20 mM by monitoring the decrease in absorption at 340 nm.

Under saturating inhibitor concentrations, the pseudo-first-order rate constant for inactivation was calculated. Alanine racemase was added to 100 μL of buffer containing 0.1 M L-Ala-P; the mixture was incubated at 37 °C for various time intervals. Activity was measured as a function of time by following the decrease in absorption at 340 nm upon dilution of 5-μL aliquots into a D-alanine assay mixture containing 0.9 mL of buffer, 20 μL of DAAO, 5 μL of LDH, 0.2 mM NADH, and 40 mM L-alanine.

Inactivation of Racemase by [^{15}N]Ala-P and Crystallization of the Enzyme–Inhibitor Complex. To 110 mg of pure racemase in 40 mL of potassium phosphate buffer (20 mM) at pH 7.4 was added 15 mg of [^{15}N]Ala-P, and activity was followed as a function of time. After 2 h, the residual enzyme activity was 0.3% and did not change after an additional 2 h. The inactive enzyme mixture was placed in a 50-mL Amicon ultrafiltration apparatus (using an Amicon PM10 membrane), and excess [^{15}N]Ala-P was removed by adding crystallizing buffer (20 mM Bis-Tris-propane, 0.2 M NaCl, and 10 mM PLP at pH 7.6), concentrating to ca. 10 mL, and repeating the addition and concentration process until all of the AlaP was removed. The inactive enzyme complex was concentrated in the crystallizing buffer to approximately 8.0 mL, giving an enzyme concentration of 15 mg/mL. PEG (2.1 g) was added and the solution stirred until all had dissolved. The solution was placed in an ice bath and the enzyme precipitated, forming microcrystals. The precipitated enzyme was collected by centrifugation at 15 000 rpm for 30 min, and the supernatant was decanted.

Solid-State ^{15}N NMR. ^{15}N MAS NMR spectra were taken on a home-built spectrometer, using aluminum oxide rotors in a double air bearing stator system (Doty Scientific, Co-

Table I: Isotropic Chemical Shifts (σ_{iso}) Derived from Solid-State MAS Spectra and Chemical Shift Tensor Elements Obtained from Rotation Sideband Analysis[a]

sample	σ_{iso}	σ_{11}	σ_{22}	σ_{33}	$\Delta\sigma$	η
[^{15}N]Ala-P, pH 7.5	1.8					
[^{15}N]Ala-P, pH 13.5	−4.7					
[^{15}N]Ala-P–PLP aldimine, pH 13.5	286.9	−33.0	294.3	599.6	469	0.95
[^{15}N]Ala-P–PLP aldimine, pH 7.5	153.9	−7.4	165.5	306.6	228	0.86
[^{15}N]Ala-P–alanine racemase complex, pH 7.5	149.9	−5.5	149.9	305.3	233	1.00

[a] All chemical shifts are in ppm, referenced to external $^{15}NH_4Cl$. A negative chemical shift value means upfield from $^{15}NH_4Cl$. Estimated errors in the σ_{ii} are ±5 ppm for the Schiff base model compounds, ±2 ppm for the enzyme–inhibitor complex, and ±0.2 ppm for MAS isotropic shifts. $\Delta\sigma$ and η are defined as follows: $\Delta\sigma = \sigma_{33} - {}^1/_2(\sigma_{11} + \sigma_{22})$; $\eta = (\sigma_{22} - \sigma_{11})/(\sigma_{33} - {}^1/_3 \text{ Tr } \tilde{\sigma})$.

lumbia, SC). Approximately 100 mg of the microcrystalline sample preparation was utilized for each NMR sample. The ^{15}N and ^{1}H Larmor frequencies were 32.2 and 317.8 MHz, respectively, with 90° ^{15}N and ^{1}H pulse lengths of ~7.5 and ~3.5 μs, respectively. Cross polarization (Pines et al., 1973) from ^{1}H to ^{15}N was used to enhance the ^{15}N signal and shorten the effective spin–lattice relaxation time. Typically, cross-polarization times were 2 ms, spinning speeds were 2–3 kHz, and recycle delays were 3–5 s. Spectra of the enzyme–inhibitor complex were recorded at low temperatures (−45 to −98 °C), whereas the model compounds were studied at room temperature.

It took approximately 16 h to obtain the ^{15}N spectrum of the Ala-P–alanine racemase complex. Isotropic chemical shifts were measured in ppm relative to external $^{15}NH_4Cl$, and no corrections were made for bulk susceptibility effects, which are expected to be small. Shift anisotropies were obtained by using a recently developed version of the Herzfeld–Berger algorithm (Herzfeld & Berger, 1980; H. de Groot, private communication).

Results and Discussion

The alanine analogue (1-aminoethyl)phosphonate has been found to inhibit the Gram-positive alanine racemase from *B. stearothermophilus* in a time-dependent, slow-binding fashion (Morrison & Walsh, 1987) with a half-life for reactivation of 25 days (Badet et al., 1986). The mechanism of inactivation has been shown to proceed via the scheme shown in Figure 1, in which inactivation (rate k_2 in Figure 1) is preceded by reversible binding (K_i in Figure 1). The values for K_i and k_2 have been determined to be 1 mM and 10 min^{-1}, respectively, at pH 7.4 (Badet et al., 1986). The pH 9.1 data reported here demonstrate that the inactivation kinetics are a strong function of pH; reversible binding (K_i) is much poorer (11 ± 1 mM) and k_2 is also substantially reduced (0.8 ± 0.1 min^{-1}). Since Ala-P is a dianion at pH 6, one may conclude that the inactivation rate is decreased by a change in the enzyme that occurs at higher pH values. This is most likely due to deprotonation of an enzyme base with an apparent pK_a of roughly 8.5 ± 0.4.

Although the kinetics of the inactivation of *B. stearothermophilus* alanine racemase with Ala-P at pH 7.4 have been carefully determined (Badet et al., 1986), knowledge of the structure of the inactive enzyme complex is incomplete. Attempts to trap an Ala-P–PLP aldimine adduct by borohydride addition were unsuccessful (Badet et al., 1986). One possibility is that Ala-P does not undergo transaldimination, but simply resides noncovalently bound in the active site. A second possibility is that the Ala-P–PLP aldimine forms, but is kinetically inaccessible to borohydride, due perhaps to an enzyme conformational change upon inactivation (i.e., isomerization of EI to EI* in Figure 1). In order to decide between these alternatives, we have performed ^{15}N NMR experiments on the enzyme–inhibitor complex and on the appropriate model compounds.

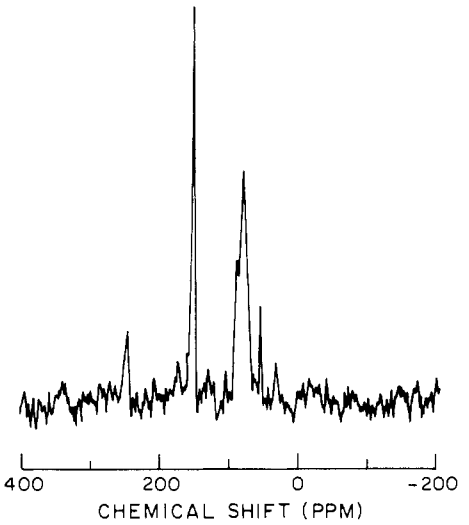

FIGURE 2: 32-MHz ^{15}N MAS spectrum of the [^{15}N]Ala-P–alanine racemase complex. The line at 80 ppm is due to the natural abundance ^{15}N in the peptide backbone, while the line at 149.9 ppm arises from the [^{15}N]Ala-P–PLP complex. Rotational sidebands from the complex are visible at ±3.1 kHz from the centerband. The isotropic chemical shift and shift anisotropy are consistent with the presence of Ala-P–PLP complex containing a protonated Schiff base linkage.

In Table I we have assembled the isotropic shifts and shift tensor values of three model compounds in which the difference between free amine and Schiff base (protonated and unprotonated) resonances alluded to above is clearly illustrated. The first compound, [^{15}N]Ala-P·HCl (the free amine model for the inhibitor), exhibits a ^{15}N isotropic chemical shift of ~2.0 ppm, and a small change in σ_{iso} is observed in going from protonated to unprotonated free amine (~7 ppm upfield). Furthermore, the ^{15}N shift anisotropy of Ala-P is quite small, less than 10 ppm, and therefore no rotational sidebands corresponding to the [^{15}N]Ala-P·HCl resonance are detectable (Harbison et al., 1981). For the case of the second model compound, [^{15}N]Ala-P–PLP aldimine at pH 13.5, the ^{15}N isotropic shift has moved considerably downfield from free Ala-P to a value of ~287 ppm. This isotropic chemical shift is characteristic of a deprotonated Schiff base linkage, a point which is further supported by the values of the shift tensor elements. For [^{15}N]Ala-P–PLP at pH 13.5, an axially asymmetric tensor (η = 0.95) with a total width of ~632 ppm is observed (Harbison et al., 1983). In contrast, the isotropic chemical shift of [^{15}N]Ala-P–PLP aldimine at pH 7.5 (~154 ppm) corresponds to that expected of a protonated Schiff base and represents a chemical shift difference between protonated and unprotonated Ala-P–PLP Schiff bases of ~130 ppm. From the data in Table I, one can also observe that the width of the chemical shift tensor narrows from ~632 to ~310 ppm upon protonation. These results clearly illustrate the sensitivity of [^{15}N]Ala-P–PLP isotropic and anisotropic chemical shifts to the state of bonding and protonation of the Ala-P nitrogen.

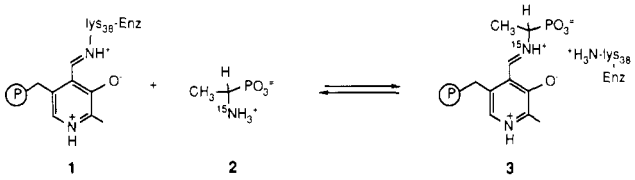

FIGURE 3: Illustration of the mechanism of inactivation of alanine racemase (**1**) by Ala-P (**2**), involving formation of the Ala-P–PLP aldimine (**3**); the position of the ^{15}N label in Ala-P and in the complex is shown.

The model compound studies thus provide the chemical shift information required for an interpretation of the solid-state ^{15}N NMR spectrum of the [^{15}N]Ala-P inactivated enzyme.

Figure 2 shows the ^{15}N MAS NMR spectrum of the gram-positive *B. stearothermophilus* racemase inactivated with [^{15}N]Ala-P. The broad ^{15}N resonance line at 80 ppm arises from the natural abundance amide nitrogens in the peptide chain backbone. The shift anisotropy of such nitrogens is small at our magnetic field and is almost completely averaged by sample spinning. This causes the majority of the ^{15}N resonance intensity to reside in the centerband (Munowitz et al., 1982). The second ^{15}N resonance from the [^{15}N]Ala-P–enzyme complex is at 150 ppm. From the model compound studies, this chemical shift indicates that the inhibitor does form a Schiff base linkage at the active site and is protonated. Analysis of the sideband intensities to determine the shift tensor elements further supports the presence of a protonated Schiff base linkage in the Ala-P–enzyme complex. The breadth of the tensor (~311 ppm) is remarkably similar to that of the model compound Ala-P–PLP aldimine at pH 7.5 (~310 ppm), and both tensors exhibit axial asymmetry (η = 1.0 and η = 0.86, respectively).

Therefore, the ^{15}N NMR results clearly show that, upon incubation with Ala-P, transaldimination of the native enzyme occurs, forming an Ala-P–PLP Schiff base linkage, as is illustrated in Figure 3. Furthermore, this suggests that the inactive enzyme undergoes a conformational change that renders the active site inaccessible to borohydride and thus prevents the reduction of the Ala-P–PLP Schiff base.

Conclusion

From the data described above, one can obtain a more detailed picture of the mechanism of inactivation of the *B. stearothermophilus* alanine racemase with the slow-binding inhibitor Ala-P. The reversible binding of Ala-P with the enzyme (K_i in Figure 2) most likely encompasses both initial noncovalent binding and transaldimination steps. Inactivation (k_2 in Figure 1) occurs with a rate constant of approximately 10 min^{-1}, which is too slow to be transaldimination (Faraci & Walsh, 1987), and may be ascribed to a change in the enzyme conformation, leading to a slowly dissociating, inactive enzyme species. It is this proposed change in the enzyme conformation that may render the active site Schiff base inaccessible to borohydride. The effect of pH on both K_i and k_2 appears to indicate that deprotonation of an enzyme residue leads to poorer binding and a lower inactivation rate. It is possible that the presence of a positive charge (from a lysine or histidine residue) is necessary for inactivation, e.g., stabilizing the phosphonate dianion form of Ala-P in the racemase active site. Phosphinic acid analogues (Badet & Walsh, 1985), which contain only a monophosphate anion at pH 7 and 9, do not lead to time-dependent, slow-binding inactivation.

With solid-state NMR, we have been able to obtain convincing evidence for the presence of aldimine in the Ala-P-inactivated alanine racemase enzyme species. In particular, low-temperature, ^{15}N MAS NMR spectra exhibit isotropic and anisotropic chemical shifts in essentially exact agreement with those obtained from Ala-P–PLP aldimine model compounds. This is the initial use of solid-state MAS NMR to elucidate the structure of an unknown enzyme–inhibitor complex, and as such it demonstrates the utility of the technique in structural investigations of high molecular weight enzymes. The approach utilized here should be generally applicable to structural studies of a wide variety of enzyme–inhibitor complexes. Furthermore, it should be possible to examine enzyme–substrate intermediates trapped at low temperature with these techniques.

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One Free Sulfhydryl Group of Plasma Fibronectin Becomes Titratable upon Binding of the Protein to Solid Substrates†

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ABSTRACT: The accessibility in human plasma fibronectin of the two free sulfhydryl groups per chain to sulfhydryl reagents 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and a maleimide derivative has been examined. For soluble fibronectin, the free sulfhydryl groups are not accessible to DTNB unless urea or guanidine hydrochloride is added [Smith et al. (1982) *J. Biol. Chem. 257*, 5831-5838]. Upon binding to polystyrene beads, 0.87 ± 0.05 sulfhydryl group per chain becomes titratable to DTNB. Experiments using fibronectin fragments demonstrate that this newly exposed sulfhydryl group is located in a Type III homologous unit between the DNA-binding and the cell-binding domains. The results suggest that, upon adsorption to solid substrates, plasma fibronectin undergoes a conformational change, thereby exposing one buried sulfhydryl group. These findings have implications regarding the "surface activation" of adhesion activities of fibronectin.

Plasma fibronectin (Fn) is a glycoprotein present in blood plasma, consisting of two subunits of about 250 kDa each linked together by two disulfide bridges near the carboxyl termini (McDonagh, 1985). The protein plays important roles in cell adhesion, wound healing, and phagocytosis. Most of these functions are expressed when Fn is attached to a surface, either solid substrates in vitro or basement membranes in vivo. For example, plasma Fn and its fragments adsorbed to polystyrene beads have been shown to promote the adhesion of fibroblast cells (McAbee & Grinnell, 1983; Schwarz & Juliano, 1984).

Human plasma Fn contains two free sulfhydryl groups per chain: one located in a Type III homologous unit between the DNA-binding and cell-binding domains (Skorstengaard et al., 1985) (designated as SH-1) and the other in the fibrin-binding domain near the carboxyl terminus (Garcia-Pardo et al., 1985) (designated as SH-2) (see Figure 2).

Previous studies have shown that the two free sulfhydryl groups in soluble Fn are not accessible to sulfhydryl reagents such as DTNB or maleimide derivatives in the absence of chaotropic agents (Smith et al., 1982; Lai & Tooney, 1984). ESR spin-label studies indicated that these two sulfhydryl groups of plasma Fn are in a cleft about 10.5 Å in length (Lai et al., 1984).

In this paper, we demonstrate that, upon binding of plasma Fn to polystyrene beads, SH-1 is exposed and becomes titratable by DTNB, while SH-2 remains buried, suggesting that plasma Fn undergoes a conformational change upon adsorption to polystyrene beads. The proximity of SH-1 to the cell adhesion domains of Fn (Skorstengaard et al., 1985) suggests that this regional change in conformation may have importance in the expression of adhesion activities of the molecule.

Materials and Methods

Materials. Tris(hydroxymethyl)aminomethane (Tris) and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma (St. Louis, MO). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and guanidine hydrochloride were purchased from Pierce (Rockford, IL). Aquacide III was obtained from Calbiochem (San Diego, CA). Polystyrene latex beads with amino groups on the surface were purchased from Polysciences (Warrington, PA); beads with diameter 0.1 or 0.5 μm were used throughout the study. According to the manufacturer's estimation, the bead suspension contains 2.5% solids. The bead concentration used in this study was calculated on the basis of this information.

Plasma fibronectin (Fn) was isolated from freshly frozen human plasma by using gelatin-Sepharose 4B affinity chromatography (Engvall & Ruoslahti, 1977). It was essentially pure as determined by 5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The 75-kDa and the C-terminal 31-kDa fragments of Fn were purified as described previously (McCarthy, 1986).

Purification of Tryptic Fragments of Fn. The fragments 34 kDa, 185 kDa, and 215 kDa were purified according to the method of Sekiguchi and Hakamori (1983), except that the separation of the 185-kDa fragment from the 215-kDa fragment was carried out at 22 °C by high-performance liquid chromatography using the Pharmacia (Uppsala, Sweden) FPLC (fast protein liquid chromatography) system equipped with a GP-250 gradient programmer. Briefly, 0.5 mL of the fragment mixture (0.6 mg/mL) in buffer A (50 mM Tris and

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