Vol. 83, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1978 Pages 107-113

ARGININE DEIMINASE: DEMONSTRATION OF TWO ACTIVE SITES AND POSSIBLE HALF-OF-THE-SITES REACTIVITY

Joachim L. Weickmann, Michael E. Himmel,
Douglas W. Smith, and David E. Fahrney

Department of Biochemistry, Colorado State University
Fort Collins, Colorado 80523

Received May 5,1978

SUMMARY

Arginine deiminase (EC 3.5.3.6) from Mycoplasma arthritidis is a dimeric enzyme. Velocity centrifugation in 6 M guanidine HCl and peptide mapping of the BrCN fragments suggest that the subunits are identical. The reaction of one out of four sulfhydryl groups with 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) has a half-life of about 30 min in 2 M guanidine HCl at 15°, pH 8. The enzyme is irreversibly inhibited by 1 mM formamidinium ion within 1 min. Inactivation by this affinity label is resolvable into two concurrent first-order reactions in the presence of guanidinium ion; the fraction of enzyme which reacts at the faster rate is about 50%. These results are interpreted as evidence for two catalytic subunits which differ in conformation.

There are two primary isoenzymes of arginine deiminase in extracts of Mycoplasma arthritidis (1). The isoenzyme from cells in log-phase growth has been shown to be a dimeric protein with a molecular weight of about 87,300 (2). Data from these studies permit the inference that the subunits are similar. For example, there is no detectable difference in SDS polyacrylamide gel electrophoresis and alanine was the only N-terminal residue found. In the present report two-dimensional peptide maps of peptides obtained by cyanogen bromide cleavage provide information that if the subunits are non-identical, the differences between them must be small. But results from this laboratory indicate the accumulation of 0.5 mol of an "amidine" intermediate per mol enzyme, a finding suggestive of only one active site (3). We have pursued this problem in more detail. The results reveal that arginine deiminase from

Abbreviations used: SDS, sodium dodecyl sulfate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); and TNB, 5-thio-2-nitrobenzoic acid.

0006-291X/78/0831-0107$01.00/0

M. arthritidis contains two active sites and exhibits an apparent "half-of-the-sites" reactivity.

METHODS

Arginine deiminase — The enzyme was prepared from M. arthritidis by the method of Weickmann and Fahrney (1). An enzyme preparation from log-phase cultures having a specific activity of 19.2 units/mg was used in this investigation. The concentration of enzyme and specific activity were determined as described previously (3).

Sedimentation studies — Arginine deiminase was dialyzed at 4° against 6 M guanidine HCl/0.1 M β-mercaptoethanol until the refractive index difference between the enzyme solution and the dialysate was due only to protein (5-6 days). This was judged from the schlieren peak area produced when the solvent was layered over the dialyzed enzyme solution in a synthetic boundary cell. Sedimentation velocities were determined in a Beckman model E ultracentrifuge, using a double sector synthetic boundary cell with sapphire windows at 42,040 rpm and 20°. Viscosities at 20° and 25° were determined using a Cannon 50M30 side-arm Ubbeholde viscometer in a water bath controlled to ±0.02°.

Peptide maps — Dialyzed samples of the protein were unfolded in 6 M guanidine HCl, reduced, carboxamidomethylated, dialyzed, lyophilized, and dissolved in cold 70% formic acid for cleavage of the methionyl peptide bonds by BrCN. Two-dimensional analysis of the resultant peptides was performed on cellulose thin layer plates (20 x 20 cm). The first dimension consisted of electrophoresis in 65% formic acid at 4° for 70 min at 500V. The plate was dried and developed at right angles in butanol/90% formic acid/water (14:7:1) at 22°. The peptides were detected with ninhydrin.

Sulfhydryl reactivity — The reaction of free -SH groups with DTNB was followed spectrophotometrically (2). Reactions were initiated by addition of 1 to 2 mg arginine deiminase.

Reaction of arginine deiminase with formamidinium ion — Kinetic studies were run in 2.0 ml total volume in 0.1 M sodium phosphate, pH 7.4, at 25°. The reaction mixture included 10 mM guanidinium chloride and 20 μg of arginine deiminase. Reactions were initiated by adding freshly prepared stock solutions of formamidinium acetate. Aliquots (0.1 ml) were mixed with 3.2 ml of standard assay medium (1) containing 6 mM arginine (K_m = 4 μM). The residual enzymic activity was assayed by measuring absorbance change at 340 nm with a Beckman Kintrac recording spectrophotometer.

RESULTS

Sedimentation studies — In earlier work the subunit structure of arginine deiminase was investigated by electrophoresis in a SDS polyacrylamide gel system (1). Both the enzyme and its reduced carboxamidomethyl derivative dissociated into a single protein band corresponding to a molecular weight of about 49,000 ± 2,000, indicating that arginine deiminase is dimeric and that interchain disulfide bonds are absent. Since this value for the apparent molecular weight is incompatible with a later value of 87,300 obtained from sedimentation equilibrium studies of the native enzyme (2), we have re-examined the molecular

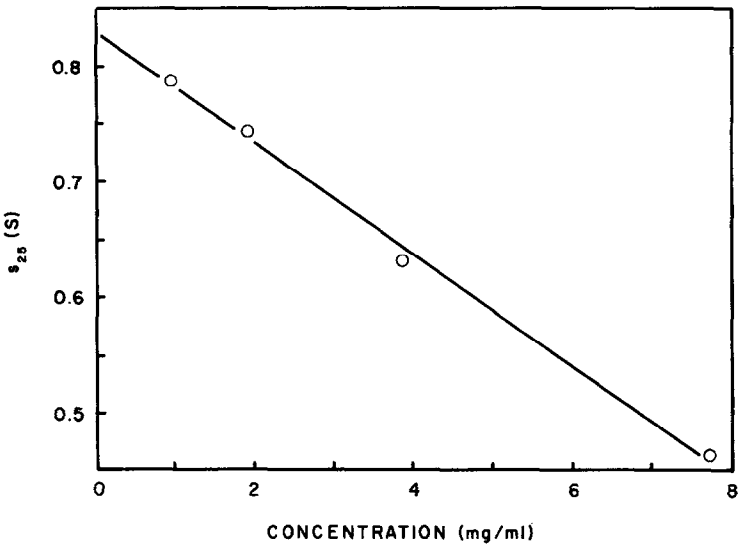
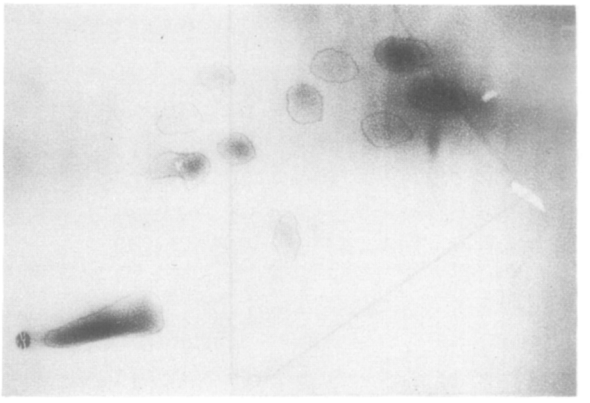


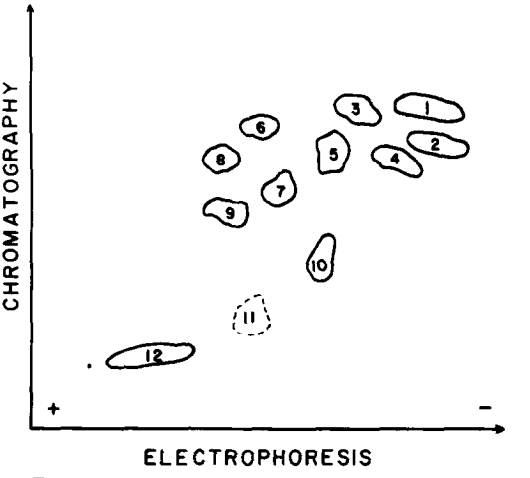
Fig. 1. Sedimentation velocity experiments with arginine deiminase in 6 M guanidine HCl/0.1 M β-mercaptoethanol: the concentration dependence of s_{25}. The sedimentation coefficients were measured at 20° using a synthetic boundary cell at 42,040 rpm and corrected to the viscosity and density of the solvent at 25°.

weight of the subunits by velocity centrifugation in guanidinium chloride/mercaptoethanol. Sedimentation coefficients were measured at four different protein concentrations (Fig. 1). Extrapolation to zero concentration gave a value of 0.83 S for s^0_{25}. The apparent number of residues per polypeptide chain was calculated from s^0_{25} by Tanford's method (4) as 398. The apparent subunit molecular weight is 44,000 ± 4,000, based on a mean residue weight of 112 (2).

Cyanogen bromide peptide maps — Performic acid oxidation of arginine deiminase has yielded a value of 19.5 mol methionine sulfone per mol of enzyme. Figure 2 shows the peptide map of arginine deiminase following separation of the BrCN fragments on cellulose thin layer plates. Twelve spots are visible, suggesting that each subunit has 11 cleavage points (or 22 mol methionine per mol enzyme). Peptide number 11 (Fig. 2) did not always run true and was often observed to have a very low color yield. Whether or not it represents an actua



A



B

Fig. 2. Peptide map of cyanogen bromide fragments of arginine deiminase. A (left) is a representative map obtained from 80 µg sample. Peptides were resolved in two dimensions as described under "Methods" and visualized by heating after immersion in 0.2% ninhydrin solution. B (right) is a composite drawing of 5 peptide maps.

BrCN cleavage product is uncertain at this time. Assuming that the methionine content of arginine deiminase is 20 residues per molecule (2.5% error in methionine sulfone analysis), complete cleavage by BrCN would be expected to yield 11 peptides if each subunit has the same primary structure.

Half-site reactivity of sulfhydryl groups — Titration of the purified enzyme with DTNB in 5 M guanidine·HCl has established the presence of 4 -SH groups per enzyme dimer (2). At pH 8, 25°, DTNB does react with the native enzyme; the rate, however, is very slow and no change in enzyme specific activity is observed. Further experiments with DTNB reveal two classes of sulfhydryl groups: (1) only two per dimer are modified in the presence of 2 M guanidine·HCl at 25°, and (2) only one -SH group reacts rapidly with DTNB at 15° in 2 M guanidine·HCl. In 2 M guanidine·HCl, 15°, the modification of one -SH group in the enzyme has a half-life of about 30 minutes. Whether further reaction with other sulfhydryl groups in the enzyme is occurring in 2 M guanidine·HCl at 15° is uncertain, since the rate of further TNB release was essentially that observed in the control (absence of protein) for hydrolysis of DTNB.

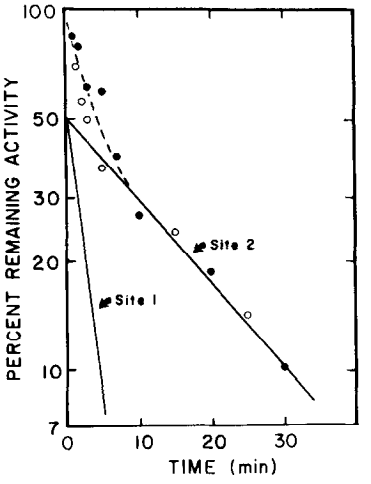


Fig. 3. The reaction of formamidinium ion with arginine deiminase at pH 7.4, 25°. Semilogarithmic plot of remaining enzyme activity against time. Data from two experiments with 0.5 mM formamidinium acetate in the presence of 10 mM guanidinium chloride are shown. The inactivation by formamidinium ion is resolvable into two concurrent first-order reactions (solid lines) in the presence of guanidinium ion; the fraction of enzyme which reacts at the faster rate (site I) is about 50%.

Both the rate of the DTNB reaction with native arginine deiminase at 25° and the rate in the presence of 2 M guanidine·HCl at 15° are independent of DTNB concentration over the range 0.3 to 1.0 mM.

Biphasic reaction of formamidinium ion with arginine deiminase — Treatment of arginine deiminase with 0.5 mM formamidinium acetate resulted in complete loss of enzymic activity at a rate too fast to monitor by conventional sampling techniques. At lower concentrations of formamidine the enzyme was not completely inactivated; e.g., at 0.1 mM formamidine only about 80% of the activity was lost, presumably because the formamidinium ion is rapidly hydrolyzed. Partially inactivated enzyme did not show any change in activity upon dialysis for several days. Evidence that derivatization occurred at the active site was two-fold: (1) in the presence of guanidinium ion, a substrate, the rate of inactivation was slowed significantly, and (2) no incorporation of ^{14}C from [^{14}C]arginine into formamidine-inactivated enzyme was observed (3).

In order to investigate the formamidine reaction at rates slow enough to

follow the reaction in more detail, arginine deiminase was inactivated by 0.5 mM formamidine in the presence of 10 mM guanidinium chloride (K_m = 10 mM). Fig. 3 is a representative first-order plot for the irreversible inactivation of the enzyme under these conditions. The nonlinear character of these plots suggests that two or more forms of the enzyme are reacting at different rates. The curve becomes "linear" after some time because the more reactive enzyme has disappeared. The final slope, therefore, gives the pseudo-first-order rate constant for the less reactive form. Subtraction of the values along the extrapolated final slope from the observed values gives a second line from which the pseudo-first-order rate constant can be calculated for the more reactive form. The intercept of the extrapolated line gives the fraction of the enzyme, about 50%, which reacts at the faster rate.

DISCUSSION

In the preceeding communication a covalent intermediate was advanced in a mechanistic proposal for catalysis by arginine deiminase (3). The formation of this intermediate involves nucleophilic attack at the sp^2 carbon atom of the guanidino group, and any substitution which enhances the electrophilicity of the guanidino group ought to enhance the rate of formation of this intermediate. Substitution of an amino group of the guanidinium ion by hydrogen, to give the formamidinium ion, causes a large change in chemical reactivity (5). In fact, we found that formamidine reacts astonishingly fast with arginine deiminase. However, the substituted enzyme does not turn over at a measurable rate. Since guanidinium ion slows the reaction, it appears that the formamidinium ion is attacking the active site.

The puzzling aspect is the biphasic manner in which formamidine-induced loss of catalytic activity occurs in the presence of guanidinium ion. There is also an unresolved question from the previous communication in regard to the accumulation of 0.5 mol of "amidine" per mol enzyme, a finding suggestive of only one active site. One explanation is that the subunits are not identical, although the evidence presented here indicates that the subunits have

identical primary structures. A second possibility is that the two subunits are identical but that the conformations of the two subunits are not identical.

The evidence to date is compatible with the idea that the dimeric enzyme is homogeneous and exhibits behavior characteristic of half-of-the-sites or half-site reactivity (6-8). Only one of the two subunits of arginine deiminase is seen in the following reactions: (1) one -SH is accessible in 2 M guanidine HCl, and (2) one [^{14}C]arginine-derived intermediate is bound to the enzyme if the steady state reaction with labeled substrate is quenched in 1 M HCl. In addition, the reaction with formamidinium ion is clearly biphasic, behavior reminiscent of similar phenomena occurring with half-site enzymes and irreversible inhibitors (6-8).

Acknowledgments — We thank Dr. Joel Pardee for his helpful advice with the peptide maps. This research was supported by Grant GM 21364 from the National Institutes of Health, USPHS.

REFERENCES

1. Weickmann, J.L., and Fahrney, D.E. (1977) J. Biol. Chem. 252, 2615-2620.
2. Weickmann, J.L., Himmel, M.E., Squire, P.G., and Fahrney, D.E. (1978) J. Biol. Chem., in press.
3. Smith, D.W., and Fahrney, D.E. (1978) Biochem. Biophys. Res. Commun., preceeding communication in this issue.
4. Tanford, C., Kawahara, K., and Lapanje, S. (1967) J. Am. Chem. Soc. 89, 729-749.
5. DeWolfe, R.H. (1975) in The Chemistry of Amidines and Imidates, Patai, S., ed., pp. 349-384, John Wiley, New York.
6. Levitzki, A., Stallcup, W.B., and Koshland, D.E., Jr. (1971) Biochemistry 10, 3371-3378.
7. Lazdunski, M. (1972) Curr. Topics Cell. Regul. 6, 267-309.
8. Seydoux, F., Malhotra, O.P., and Bernhard, S.A. (1974) CRC Crit. Revs. Biochem. 2, 227-257.