Arginine Deiminase from *Mycoplasma arthritidis*

PROPERTIES OF THE ENZYME FROM LOG PHASE CULTURES*

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Hydrodynamic, chemical, and optical properties of arginine deiminase (EC 3.5.3.6) from *Mycoplasma arthritidis* are reported for the enzyme isolated from log phase cells. The $s_{280}$ and $D_{280}$ values of the enzyme are 5.48 S and 5.87 × 10⁻⁴ cm²/s, respectively; the molecular weight is 87,300. Determination of the amino acid composition shows that about 45% of the residues are non-polar. Another unique feature of the composition is the presence of 36 half-cystine residues. The state of oxidation of the half-cystines appears to be well established as 16 disulfide and 4 sulfhydryl groups. The reaction of 1 sulfhydryl group with 0.3 $\times 10^{-7}$ CM 5,5'-di-thiobis(2-nitrobenzoic acid) has a half-life of about 50 min at pH 8. The modified enzyme retains full activity. Two —SH groups are accessible to this reagent in 2 M guanidine hydrochloride, whereas all 4 —SH groups react immediately in 4 M guanidine hydrochloride. Reduction of disulfide bonds with dithiothreitol occurs only to a limited extent in 8 M urea, but is complete in 4 M guanidine hydrochloride. The enzyme loses activity immediately at pH 2.5, but retains full activity upon standing 8 h at pH 9.5 in several buffers. The large number of cystine residues leads to a complex near ultraviolet circular dichroism spectrum with cystine contributions superimposed on contributions from aromatic residues. The far ultraviolet spectrum suggests that the molecule contains about 18% $\alpha$ helix. At pH 2.5, $\beta$ conformation and disulfide contributions are dominant. Aromatic and $\alpha$ helix bands are reduced considerably at pH 9.5.

Very little is known about arginine deiminase, an enzyme which catalyzes the irreversible hydrolysis of arginine to citrulline and ammonia. The enzyme has been found in a number of species among the bacteria (1-12), mycoplasmas (13, 14), unicellular green algae (15, 16), and yeast (17), as well as the protozoan *Tetrahymena pyriformis* (18). Arginine deiminase of high purity has been prepared in a number of laboratories. Kihara and Snell (2) and Petrack et al. (3) have reported as the protozoan *Tetrahymena pyriformis* (18). Arginine deiminase is the first enzyme in the pathway in cells where arginine degradation proceeds to ornithine via citrulline and ammonia. The enzyme has been found in a number of species among the bacteria (1-12), mycoplasmas (13, 14), unicellular green algae (15, 16), and yeast (17), as well as the protozoan *Tetrahymena pyriformis* (18). Arginine deiminase of high purity has been prepared in a number of laboratories. Kihara and Snell (2) and Petrack et al. (3) have reported as

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Enzyme Activity and Purity—We have taken advantage of our previous studies (19) on arginine deiminase from *M. arthritidis* to obtain preparations of the enzyme which are apparently homogeneous in two steps, protamine sulfate fractionation and DEAE-agarose chromatography. The methods applied in establishing the degree of purity of our preparations include: (a) polyacrylamide gel electrophoresis, both in pH 7.8 and pH 9.5 buffers and in 0.1% sodium dodecyl sulfate, (b) 5,5'-di-thiobis(2-nitrobenzoic acid) has a half-life of about 50 min at pH 8. The modified enzyme retains full activity. Two —SH groups are accessible to this reagent in 2 M guanidine hydrochloride, whereas all 4 —SH groups react immediately in 4 M guanidine hydrochloride. Reduction of disulfide bonds with dithiothreitol occurs only to a limited extent in 8 M urea, but is complete in 4 M guanidine hydrochloride. The enzyme loses activity immediately at pH 2.5, but retains full activity upon standing 8 h at pH 9.5 in several buffers. The large number of cystine residues leads to a complex near ultraviolet circular dichroism spectrum with cystine contributions superimposed on contributions from aromatic residues. The far ultraviolet spectrum suggests that the molecule contains about 18% $\alpha$ helix. At pH 2.5, $\beta$ conformation and disulfide contributions are dominant. Aromatic and $\alpha$ helix bands are reduced considerably at pH 9.5.

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TABLE I

Comparison of kinetic parameters for reaction of M. arthritidis arginine deiminase with L-arginine under different conditions

<table>
<thead>
<tr>
<th>Assay</th>
<th>pH</th>
<th>Temperature</th>
<th>V</th>
<th>Kₐ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrulline</td>
<td>6.5°</td>
<td>37</td>
<td>53</td>
<td>100-400</td>
<td>14</td>
</tr>
<tr>
<td>Citrulline</td>
<td>6.5°</td>
<td>37</td>
<td>55</td>
<td>N. D.</td>
<td>19</td>
</tr>
<tr>
<td>Ammonia</td>
<td>7.2°</td>
<td>25</td>
<td>50</td>
<td>N. D.</td>
<td>19</td>
</tr>
<tr>
<td>Ammonia</td>
<td>7.2°</td>
<td>37</td>
<td>42.8</td>
<td>28</td>
<td>19</td>
</tr>
</tbody>
</table>

* Potassium phosphate, 0.1 M.
* N. D., not determined; see text.
* Tes, 50 mM; ionic strength adjusted to 50 mM with NaCl.

Additional evidence is presented in the miniprint supplement.

Eleven preparations obtained from DEAE-agarose chromatography have exhibited specific activities ranging from 16 to 20 units/mg under standard conditions used in the spectrophotometric assay (pH 7.2, 25°C). This assay is a continuous rate method based on the reductive amination of α-ketoglutarate catalyzed by glutamate dehydrogenase. The maximum specific activity observed in our laboratory for arginine deiminase I (the enzyme from log phase culture) is 20.4 units/mg using the spectrophotometric assay (Table I). This value is slightly higher than that reported in our early paper (19), but this is due to the fact that E₅₆₀ for estimating protein concentration has been revised from 10.0 to 10.9 in the present work. It is worthy of notice that the specific activity cannot be determined under optimal conditions by means of the spectrophotometric assay because of limitations imposed by the coupling enzyme glutamate dehydrogenase. Table I also shows the results reported by Schimke et al. (14) for the enzyme from the same organism (M. arthritidis 07, formerly reported as M. hominis 07). The maximal specific activity is 55 units/mg under the conditions of the citrulline assay (pH 6.5, 37°C). This value agrees with that reported by Schimke et al. (14).

Although the citrulline assay can be used to measure V under optimal conditions (pH 6.5, 37°C), this fixed-time assay lacks the sensitivity and precision required for an accurate determination of the Kₐ.

**Titration of Sulfhydryl Groups**—The role of cysteine residues of the enzyme has been investigated with the sulfhydryl reagent DTNB. Sulfhydryl group titrations in denaturing solvents such as 5 M guanidine hydrochloride have established the presence of 3.9 ± 0.1 (S.D.) (10 determinations) —SH groups/87,300 g of protein. However, further studies with DTNB, presented in Fig. 1, reveal two classes of sulfhydryl groups. At pH 8 DTNB does react with the native enzyme; however, the rate is very slow and whether the reaction continues to completion is uncertain. There is no significant change in enzyme activity associated with the modification of one to two sulfhydryl groups, although there is a tendency for the enzyme to lose 5 to 10% activity under the conditions used in this experiment. The rate of the DTNB reaction with native enzyme is independent of DTNB concentration over the range 0.1 to 1.0 μM, suggesting that the rate being monitored is the rate at which the enzyme undergoes a conformational change exposing the —SH groups to DTNB. This is in agreement with experiments in which concentrations of guanidine hydrochloride or urea were varied. The effects of guanidine hydrochloride are illustrated in Fig. 1a. Two molar guanidine hydrochloride stabilizes a conformation of the protein in which 2 mol of —SH are available for titration. An increase in the concentration of denaturant from 3.5 to 4.0 M causes a large increase in reactivity toward DTNB, indicating that unfolding of the protein is essentially complete in 4 M guanidine hydrochloride. The same effects are noted in urea as the denaturant (Fig. 1b), even though urea is generally less effective in causing protein unfolding. Only two sulfhydryl groups are accessible to DTNB in 2 M urea and a noticeable change occurs between 3 and 4 M urea. However, the rate of reaction in 8 M urea is

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The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); Tes, N-[tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid; SDS, sodium dodecyl sulfate.
Properties of Mycoplasmal Arginine Deiminase

The rate of reaction in different concentrations of SDS (Fig. 1c) is also slow and no separation of the two classes of sulphydryl groups is observed.

**Optical Properties of Arginine Deiminase**

Far ultraviolet circular dichroism spectra of the enzyme as a function of pH are presented in Fig. 2A. The native enzyme at pH 7.0 exhibits negative maxima in the peptide region at 220 and 211 nm. The α helix content was calculated by the method of Chen et al. (21). A value of 18% was found, based on a mean residue ellipticity of $-7.8 \times 10^2$ degrees cm$^2$ dmol$^{-1}$ at 222 nm. At pH 9.5 no wavelength shifts of maxima occurred, although the protein apparently loses much of its helical content, as the calculated value for α helix was 5%. At pH 2.5 a maximum at 218 nm with a shoulder at 208 nm appears, indicating that $\beta$ conformation now dominates the remaining structure.

In the near ultraviolet region (Fig. 2B), the native enzyme at pH 7.0 displays two negative circular dichroism maxima at 263 and 268 nm, which have been assigned in bovine pituitary growth hormone to phenylalanine (22). Of interest is the broad negative band occurring at 295 nm with a molar ellipticity of $-2.4 \times 10^4$ degrees cm$^2$ dmol$^{-1}$. Considering the large number of disulfides present in the native enzyme, i.e. 16, this band might be assigned to the long wavelength contribution of a small population of strained disulfides. However, this is unlikely, as dihedral angles for the observed wavelengths of the lowest energy transition would be near 55° (23). This apparently red-shifted maximum may also be due to the superposition of a positive tyrosine or tryptophan band, occurring as a shoulder at 284 nm, and a negative disulfide band. The dihedral angles of the disulfides may then be normal, i.e. between 60 and 90°, with a CD maximum at wavelengths below 290 nm. Still, the possibility exists that the $\beta$ transition of tryptophan residues buried in a strongly hydrophobic pocket may be giving rise to this band, as seen in lysozyme (24) and in bovine pituitary growth hormone (22). The decrease in molar ellipticity at pH 9.5 was seen in all CD bands except the negative maxima at 263 and 268 nm. Although the possibility of disulfide interchange at this pH exists, arginine deiminase retains full activity when assayed at pH 7.0 following incubation at pH 9.5 for 8 h. The near ultraviolet circular dichroism at pH 2.5 shows a broad band centered near 270 nm with two small negative maxima superimposed at 275 and 279 nm. These two negative maxima may be due to tyrosine residues as indicated in the study on ovine interstitial cell stimulating hormone by Bewley et al. (25). It would appear that most of the side chain CD at low pH is dominated by the disulfide contribution. Essentially all aromatic CD at wavelengths above 290 nm is lost when arginine deiminase is partially unfolded at pH 2.5.

**DISCUSSION**

The reason for the discrepancy between the hydrodynamic data reported here and those of Schimke et al. (14) is of considerable interest, but unclear at the moment. The possibility that the discrepancy results from experimental error seems unlikely, but cannot be dismissed. To facilitate comparison, we have converted the published sedimentation and diffusion coefficients from 25 to 20°C. The values $s_{20,w} = 5.03$ S, $D_{20,w} = 5.91 \times 10^{-7}$ cm$^2$/s, $\bar{v}$ (assumed) = 0.73, $M_r = 78,300$, and $f/f_0 = 1.28$ may then be directly compared with our values: $s_{20,w} = 5.48$ S, $D_{20,w} = 5.87 \times 10^{-7}$ cm$^2$/s, $\bar{v}$ (from amino acid composition) = 0.736, $M_r = 87,300$, and $f/f_0 = 1.22$. Three factors contribute to the 11% discrepancy in molecular weight: the differences in $\bar{v}$ (about 1% of the discrepancy), the diffusion coefficient (about 1%) and the sedimentation coefficient (about 8%).

Furthermore, Schimke et al. (14) report negligible circular dichroism spectra of the enzyme as a function of pH are presented in Fig. 2A. The native enzyme at pH 7.0 exhibits negative maxima in the peptide region at 220 and 211 nm. The α helix content was calculated by the method of Chen et al. (21). A value of 18% was found, based on a mean residue ellipticity of $-7.8 \times 10^2$ degrees cm$^2$ dmol$^{-1}$ at 222 nm. At pH 9.5 no wavelength shifts of maxima occurred, although the protein apparently loses much of its helical content, as the calculated value for α helix was 5%. At pH 2.5 a maximum at 218 nm with a shoulder at 208 nm appears, indicating that $\beta$ conformation now dominates the remaining structure.

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Furthermore, Schimke et al. (14) report negligible
concentration dependence of $s$ while we found the concentration dependence to be that expected for a nonassociating globular protein. Thus, if the cause for the discrepancy is experimental error, the determination of the dependence of $s$ on $c$ is probably the most likely source.

Although there is a possibility that the two laboratories may be working with different strains of $M$. arthritidis, it seems unlikely that this could account for the discrepancy in the hydrodynamic data. The procedure developed by Schimke et al. (14) for purification of arginine deiminase from $M$. arthritidis 07 can be used with other species of Mycoplasma (26). Furthermore, experiments in our laboratory have demonstrated that the enzyme forms isolated from the 07 and ATCC 14152 strains are indistinguishable by DEAE-agarose chromatography.

The remaining possibility and the one to which we are most attracted, is that the hydrodynamic data from both laboratories are correct and that arginine deiminase I (87,300 daltons) characterized in this study loses a component (subunit or fragment) of about 9000 daltons during the conversion to deiminase III (78,300 daltons) which appears to be identical to the form characterized by Schimke et al. (14), as pointed out in the introduction to the text. If this hypothesis is correct, we still have much to learn about the conformation and subunit structure of the native enzyme.

In regard to the chemical properties of the mycoplasmal arginine deiminase, one outstanding feature is the presence of 30 half-cystine residues. The state of oxidation of the half-cystines in this enzyme appears to be well established as 16 disulfide bonds and four sulphydryl groups. The large number of cystine residues leads to a complex near ultraviolet CD spectrum and undoubtedly contributes significantly to the thermostability of the enzyme, as well as its resistance to unfolding in denaturing solvents. Another remarkable characteristic of the enzyme from a compositional viewpoint is a very high content of nonpolar amino acids. The ratio of polar to nonpolar residues, as defined by Hatch (27), is 1.2. Extracellular, cytoplasmic, and integral membrane proteins have polar:nonpolar ratios ranging from 1 to 3; however, this ratio is 1.0 to 1.2 for most integral membrane proteins (28). Since there is no evidence that arginine deiminase resides within the cell membrane, a low value of the polar:nonpolar ratio is interesting. Perhaps the high content of nonpolar residues in the mycoplasmal enzyme is linked to its exceptionally high cystine content. Anfinsen (29) has noted a marked tendency for disulfide bonds to be buried in hydrophobic regions.

Perhaps the most remarkable feature of the mycoplasmal enzyme is its extraordinarily high content of cystine bonds. Most intracellular proteins do not form disulfide bonds but retain free sulphydryl groups; disulfide bonds exist primarily in proteins which function under extracellular conditions (29). This generalization does not encompass extracellular enzymes of bacteria since amino acid analyses of a large number of proteins secreted by bacteria have established the absence of half-cystine in most of these proteins (30). Thus the high presence of a small fraction of irreversibly associated protein. A value for $M$ of 87,000 was obtained by extrapolation to zero concentration.  

Properties of Mycoplasmal Arginine Deiminase

REFERENCES

Additional references are found on p. 6015.
properties of mycoplasmal arginine deiminase

by jeanette l. henschen, michael l. henschen, philip s. seipp, and david l. henschen

experimental procedures

Chemicals - naphthylamine (cas 68-80-8) 100 to 300 mesh, exchange capacity 315 meq/g (as supplied by sepp-v laboratories, severance, ky), was used for quantitative determination of the exchange capacity of the purified protein preparation. the protein was obtained from c. pylori strain hmr6014 using a modification of the method of kegel and alain (6). the purified protein was stored at 4° c in 50 mm sodium phosphate buffer, 0.15 m sodium chloride, 2% sucrose, and 0.02% sodium azide. the purified protein was used for molecular weights standards were the same as described previously (6). the presence of the arginine deiminase activity assay, except l-arginine, was purchased from sigma.

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method a - the assay mixture for the arginine deiminase activity assay was prepared as follows: 200 u of purified protein was added to a reaction mixture containing 100 mm sodium phosphate buffer, 50 mm sodium chloride, 0.001 m l-arginine, and 0.001 m sodium acid. the reaction was initiated by adding 0.1 m l-arginine to the assay mixture. the change in absorbance was monitored at 280 nm.

method b - the assay mixture for the arginine deiminase activity assay was prepared as follows: 200 u of purified protein was added to a reaction mixture containing 100 mm sodium phosphate buffer, 50 mm sodium chloride, 0.001 m l-arginine, and 0.001 m sodium acid. the reaction was initiated by adding 0.1 m l-arginine to the assay mixture. the change in absorbance was monitored at 280 nm.

results

enzyme stability - arginine deiminase has been found to be stable when stored at 4° c for 10 to 30 days. this enzyme was found to be stable for at least 6 months. incubation at 50° c for 10 min resulted in a 50% decrease in enzyme activity.

ammonia liberation - the ammonia liberation assay was performed as follows: 200 u of purified protein was added to a reaction mixture containing 100 mm sodium phosphate buffer, 50 mm sodium chloride, 0.001 m l-arginine, and 0.001 m sodium acid. the reaction was initiated by adding 0.1 m l-arginine to the assay mixture. the change in absorbance was monitored at 280 nm.
Properties of Mycoplasmal Arginine Deiminase

1061

Molecular weight of gel filtration — The molecular weight of arginine deiminase was estimated by gel filtration on a column of Sephacryl S-300. A calibration curve was constructed with maltose, bovine serum albumin, ovalbumin, and the antibiotics and vitamins listed above as molecular weight standards. The molecular weight of arginine deiminase was determined from its elution volume with respect to the standard protein peak with a partition coefficient corresponding to a molecular weight of 60,000 ± 1,000. This value is significantly lower than that of 131,000 obtained by gel filtration on a Sephadex G-100 column (1).

Anticatalytic properties — The anticatalytic properties of arginine deiminase were examined earlier (1). This enzyme has the ability to inhibit the activity of arginine deiminase for a protein isolated from Mycoplasma. The analysis presented in Table I shows the decrease in activity of arginine deiminase for a protein isolated from Mycoplasma after incubation with 1% formaldehyde solutions at 37° for 30 min. The results show that the activity of arginine deiminase decreases with time, which is consistent with the finding that the activity of arginine deiminase decreases with time in experiments involving formaldehyde (19). Therefore, the anticatalytic properties of arginine deiminase are conserved in the presence of formaldehyde solutions.

The molecular weight of arginine deiminase was determined by gel filtration on a column of Sephacryl S-300. A calibration curve was constructed with maltose, bovine serum albumin, ovalbumin, and the antibiotics and vitamins listed above as molecular weight standards. The molecular weight of arginine deiminase was determined from its elution volume with respect to the standard protein peak with a partition coefficient corresponding to a molecular weight of 60,000 ± 1,000. This value is significantly lower than that of 131,000 obtained by gel filtration on a Sephadex G-100 column (1).
Arginine deiminase from Mycoplasma arthritidis. Properties of the enzyme from log phase cultures.

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