Inhibition of *E. coli* L-Asparaginase by Reaction with 2,3-Butanedione. Chemical Modification of Arginine and Histidine Residues

Dorothee Petz, Hans-Gerhard Löffler, and Friedh. Schneider

Physiologisch-Chemisches Institut der Universität Marburg/Lahnberge, D-3550 Marburg

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E. coli Asparaginase, 2,3-Butanedione, Arginine and Histidine Residues

The inactivation of *E. coli* asparaginase by 2,3-butanedione studied with L-asparagine and diazooxonorvaline as substrates obeys pseudo first order kinetics. Activity losses are linear with respect to arginine and histidine modification, with complete inactivation being correlated with alteration of one arginine and one histidine per subunit. The rate of inactivation of the enzym was reduced in the presence of competitive inhibitors like L-2-amino-2-carboxyethane-sulfonamide. Under comparable conditions 1,2-cyclo hexanedione does not affect the activity of L-asparaginase.

Since it is well established that only substrate analogues with a free carboxyl group are accepted as substrates or competitive inhibitors by L-asparaginase from *E. coli* [1, 2], we have examined by chemical modification with 2,3-butanedione [3, 4] the possible functional role of arginine residues, which appear to be involved in substrate binding in may enzymes as anion recognition site [5 – 7]. 2,3-butanedione in borate buffer is considered to be a highly selective modification reagent for arginine residues [3, 4]. In the course of these experiments we observed, however, a loss of arginine *and* histidine residues following the treatment with this modification reagent under usual conditions. This modification eliminated the activity of the enzyme totally after alteration of one arginine and one histidine. In the present communication we report this unexpected result.

Materials and Methods

E. coli asparaginase (spec. act. 220 U/mg) was a gift of Bayer, Elberfeld. 2,3-butanedione, L-valine and 1,2-cyclohexanedione were from Merck, Darmstadt. 2,3-butanedione was redestilled every two weeks. L-2-amino-2-carboxy-ethane-sulfonamide [8], diazooxonorvaline [9] and DL-N-ethyl-asparagine [10] were synthesized as described in the references cited.

Assay of the enzyme

The relative activity of the enzyme was assayed by conductivity measurements with a Digitalmeter Digi 610, WTW, Weilheim: 10 – 20 µl of the enzyme solution were mixed with 3 ml of 1 mM asparagine in 1 mM Trisbuffer pH 7.3. The increase of conductivity with time was recorded with a Servogor S Metra-Watt, Nürnberg. In some cases the rate of formation of ammonia from asparagine was controlled enzymatically [11] or as described by Berthelot [12]. In parallel experiments the rate of asparaginase catalyzed decomposition of diazooxonorvaline was measured spectrophotometrically at 274 nm as described elsewhere [13]. Protein concentration of asparaginase was assayed spectrophotometrically at 278 nm using $A\,|^{\%}_{cm} = 7.55$ [14].

Modification of L-asparaginase with 2,3-butanedione and 1,2-cyclohexanedione

1 ml samples of a 2 – 5 µM solution of the enzyme in 50 mM borate buffer pH 7.6 were incubated at 20 °C with 5 – 50 µl of a 1 M solution of 2,3-butanedione in the same buffer. The final concentration of the modification reagent was 5.5 – 55 mM. Samples of these incubates were withdrawn at timed intervals and the activity of the enzyme was assayed between 0 and 80 min. Parallel kinetic runs were conducted with the unmodified enzyme.

Modification experiments with 1,2-cyclohexanedione were performed in the same way. The concentration of the modification reagent was 20 – 100 mM. Additional experiments were done at pH 8.5.

Amino acid analyses

The extent of arginine and histidine modification was estimated by determining the number of intact arginine and histidine residues in the enzyme by

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amino acid analyses: Samples of the incubation mixture containing 0.5 mg of the modified enzyme were withdrawn and worked up as described in refs. [3] and [15]. Both methods gave the same results.

a) The reaction was stopped by mixing the sample with 50% cold acetic acid. Thereafter it was dialyzed for 10 h against 10% and 5% acetic acid. The solution was lyophilized and the protein was hydrolyzed in 6 N HCl/0.5% thioglycolic acid for 22 h at 110 °C. The solutions were freed from oxygen by bubbling argon through the samples before sealing. b) The modification reaction was stopped by addition of 0.2 ml 6 N HCl to the sample withdrawn from the reaction mixture. The protein precipitate was separated by centrifugation and dissolved for hydrolysis in 1 ml 6 N HCl as described.

The arginine content of the native enzyme was taken as 32 mol/mol enzyme and the histidine content as 12 mol histidine/mol enzyme [16].

Results

Treatment of *E. coli* asparaginase with 2,3-butanedione in borate buffer gives rise to a loss of activity of the enzyme which is not caused by a dissociation into subunits, as could be demonstrated by polyacrylamide gel electrophoresis. The semilogarithmic plot demonstrates that the inactivation rates follow pseudo first order kinetics with the rate of inactivation being dependent upon the concentration of 2,3-butanedione and the borate concentration (Fig. 1). In the concentration range between 2 – 6 μM asparaginase the pseudo first order rate constants were independent from the enzyme concentration. The activity of the native control remained constant during the reaction time. Long term incubation with all 2,3-butanedione concentrations tested produced complete inactivation of the enzyme. Exactly the same results were obtained with diazooxonorvaline as substrate for the determination of the enzyme activity (data not shown).

The order of inactivation with respect to 2,3-butanedione concentration was determined by the method of Levy *et al.* [17] from a plot of log of reciprocal of the half-times versus log concentration of the inactivator (Fig. 2). The points fit a straight line with a

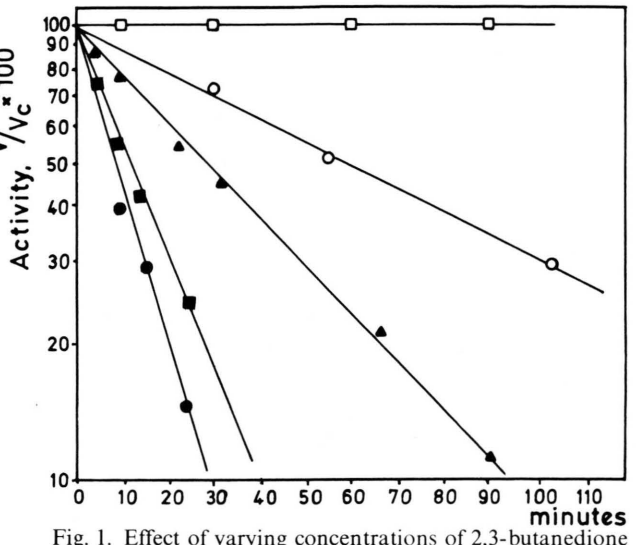

Fig. 1. Effect of varying concentrations of 2,3-butanedione on the activity of *E. coli* asparaginase as a function of time. Enzyme was incubated with 9.6 mM – ○ –, 24.4 mM – ▲ –, 38.5 mM – ■ –, 47.6 mM – ● – 2,3-butanedione in 50 mM borate buffer pH 7.6, 20 °C. Native enzyme alone in borate buffer – □ –. Enzyme concentration 5 μM. The corresponding pseudo first order rate constants of the inactivation are 0.012 min^{-1}, 0.025 min^{-1}, 0.058 min^{-1} and 0.077 min^{-1}.

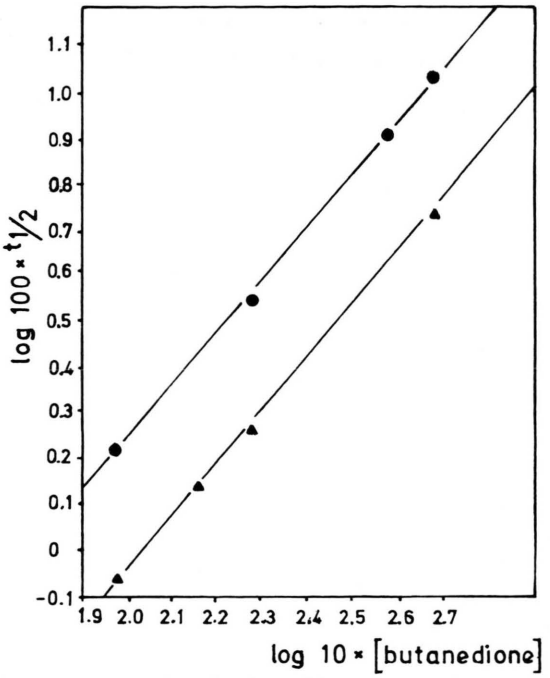

Fig. 2. Order of inactivation with respect to the cocentration of 2,3-butanedione. Plot of log $1/t_{1/2}$ versus log concentration of the modification reagent. The slope of the plot (1.17) equals the order of the reaction. Conditions see Fig. 1. ●——● reaction in the absence, ▲——▲ in the presence of 10 mM of the competitive inhibitor L-2-amino-2-carboxyethane-sulfonamide.

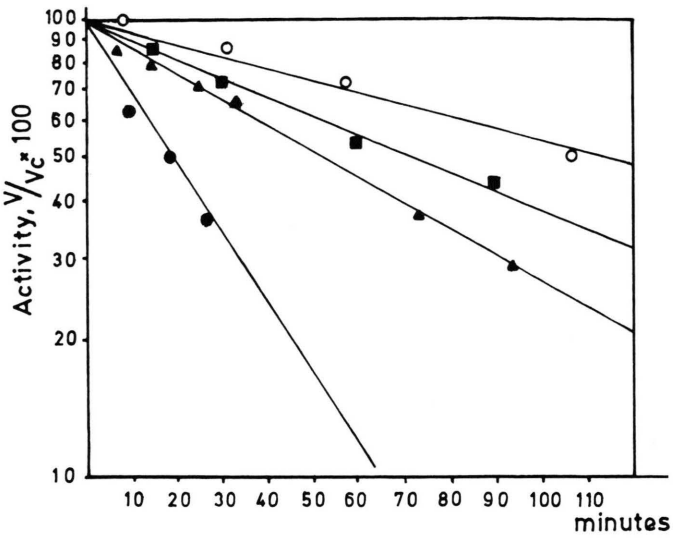

Fig. 3. The same measurements as in Fig. 1 in the presence of 10 mM of the competitive inhibitor L-amino-2-carboxyethane sulfonamide. The first order rate constants of inactivation were (2,3-butanedione concentration in parenthesis) 0.039 min^{-1} (47.6 mM) ●, 0.013 min^{-1} (19.2 mM) ▲, 0.010 min^{-1} (14.6 mM) ◆, 0.006 min^{-1} (9.6 mM) ○.

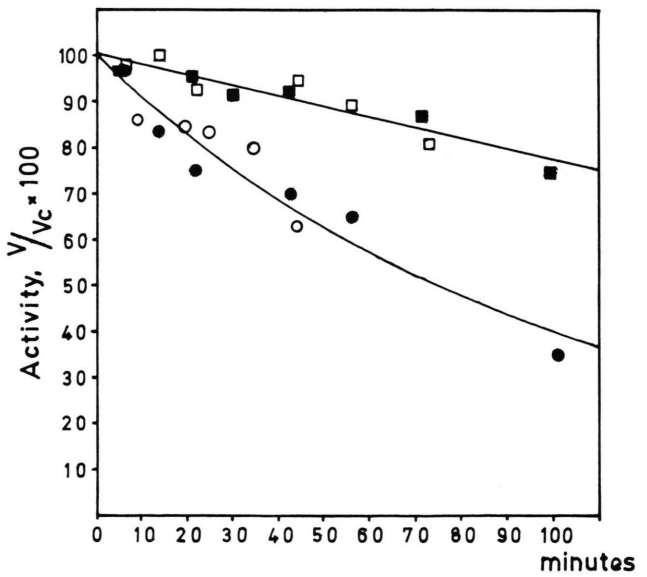

Fig. 4. Rate of inactivation of *E. coli* asparaginase by 2,3-butanedione with L-asparagine – ○ – and dioxoazonorvaline – ● – as substrate. Conditions: 9.6 mM 2,3-butanedione, 50 mM borate buffer pH 7.6, 20 °C; activity with asparagine □ and diazooxonorvaline ■ in the presence of 10 mM sulfonamide.

slope equal to 1.17. This value indicates the binding of about one molecule of 2,3-butanedione per subunit as inactivation occurs. This method gives no information on the modification of amino acid residues which are not involved in the activity of the enzyme.

The protective effect of 10 mM of the competitive inhibitor L-2-amino-2-carboxy-ethane-sulfonamide ($K_i = 9.5 \times 10^{-5}$ [2]) against the inactivation of asparaginase by different concentrations of 2,3-butanedione is demonstrated in Fig. 3. In the presence of this sulfonamide the rate of inactivation is reduced to about half of the values found without the inhibitor.

The order of inactivation with respect to 2,3-butanedione concentration in the presence and absence of the competitive inhibitor is illustrated in Fig. 2; the inhibitor has no effect on the order of the inactivation reaction.

The sulfonamide afforded protection against the inactivation of the enzyme by 2,3-butanedione measured with the two substrates asparagine and dioxo-

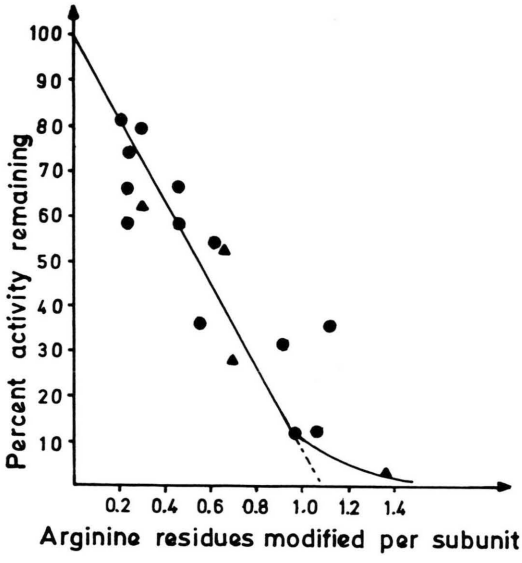

Fig. 5. Correlation between residual activity (in percent) and loss of arginine residues per subunit during inactivation of L-asparaginase by 24.4 mM – ● – or 47.6 mM – △ – butanedione in 50 mM borate buffer pH 7.6, 20 °C.

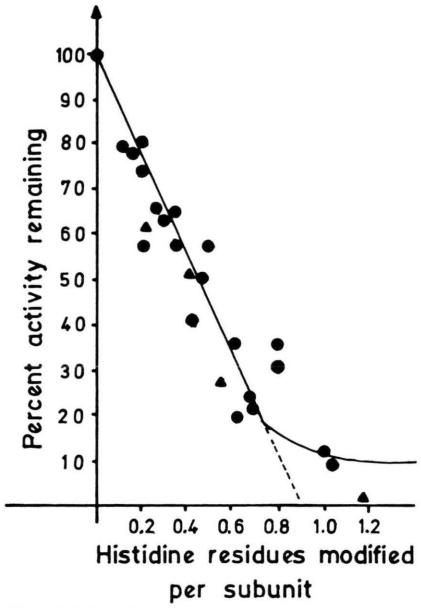

Fig. 6. Number of histidine residues modified during inactivation of *E. coli* asparaginase with 2,3-butanedione. Conditions see Fig. 5.

azonorvaline (Fig. 4). This observation suggests that both activities, the catalysis of the hydrolysis of asparagine and the catalysis of the decomposition of diazooxonorvaline take place at the same active site.

Protection of the enzyme against the inactivation by 2,3-butanedione could also be afforded by other competitive inhibitors. In the presence of 10 mM L-valine, D-asparagine ($K_i = 1.9 \times 10^{-4}$ M) and DL-ethylasparagine ($K_i = 2 \times 10^{-4}$ M) the rate constants of inactivation were found to be 0.015 min^{-1}, 0.020 min^{-1} and 0.019 min^{-1}.

The correlation between residual activity and extend of modification of arginine residues per subunit is illustrated in Fig. 5. A linear relationship between the loss of activity of asparaginase and the modification of arginine residues was found. Modification of about one arginine per subunit out of 8 present gives rise to complete inactivation of the enzyme.

The amino acid analyses of the modified enzyme preparations revealed further a loss of histidine, while no alteration of lysine residues could be found. This result was rather unexpected, because 2,3-butanedione is considered to be a highly specific reagent for the modification of arginyl residues. The correlation between loss of activity and degree of alteration of histidine is demonstrated in Fig. 6. Catalytic activity is eliminated after loss of about one histidyl residue per subunit. Other amino acids are not affected by the treatment of the enzyme with 2,3-butanedione.

Discussion

The most surprising result of the present experiments is the loss of arginyl *and* histidyl residues after treatment of *E. coli* asparaginase with 2,3-butanedione in borate buffer, a reagent known to be highly specific for the modification of arginine. At the moment we have no idea on the mechanism of the elimination of histidine residues by reaction of the enzyme with 2,3-butanedione. We would like to remember, however, that this is not the first example of absolutly unexpected loss of amino acids observed in the course of modification experiments with proteins [18]. The question whether the alteration of arginine or histidine is responsible for the loss of activity of the protein may be answered by the following consideration.

From Fig. 2 it becomes evident that binding of about one molecule of 2,3-butanedione per subunit abolishes the catalytic activity. The amino acid analyses of the modified enzyme revealed however a loss

of arginine and histidine. Studies on the mechanism of *E. coli* asparaginase by different authors have shown that histidine residues are part of the catalytic or binding site of the enzyme [19 – 22]. From photooxidation experiments [19] and modification with diethylpyrocarbonate [20] it is concluded, that 2 histidine residues per subunit are closely associated with enzymic action. Jaenicke *et al.* found, that modification of 2 tyrosine and 1 histidine per subunit totally eliminated catalytic activity [22]. Even if the modified arginine residues are not involved in the binding of substrates and competitive inhibitors, inactivation of the enzyme is to be expected already by the loss of the histidine residues. From the present experiments we therefore suggest that arginine is not essential for the activity of asparaginase and the inactivation is a consequence of the alteration of histidine residues.

[1] K. H. Röhm and Fr. Schneider, Hoppe-Seyler's Z. Physiol. Chem. **352,** 1739 – 1743 (1971).
[2] V. Herrmann, K. H. Röhm, and Fr. Schneider, FEBS Letters **39,** 214 – 217 (1974).
[3] J. F. Riordan, Biochemistry **12,** 3915 – 3922 (1973).
[4] C. L. Borders jr. and J. F. Riordan, Biochemistry **14,** 4699 – 4704 (1975).
[5] J. F. Riordan, K. D. McElvany, and C. L. Borders jr., Science **195,** 884 – 886 (1977).
[6] L. G. Lange, J. F. Riordan, and B. L. Vallee, Biochemistry **13,** 4361 – 4370 (1974).
[7] Fr. Schneider, Naturwissenschaften **65,** 376 – 381 (1978).
[8] H. Heymann, T. Ginsberg, Z. R. Gulick, E. A. Konopka, and R. L. Mayer, J. Amer. Chem. Soc. **81,** 5125 – 5128 (1959).
[9] Y. Liwschütz, R. D. Irsay, and A. J. Vincze, J. Chem. Soc. **1959,** 1308 – 1311.
[10] N. De Groot and N. Lichtenstein, Biochim. Biophys. Acta **40,** 92 – 98 (1960).
[11] Fr. da Fouseca-Wollheim, H. U. Bergmeyer, and J. Gutmann, Methoden der Enzymatischen Analyse, (H. U. Bergmeyer, ed.) 3. edition, **Vol. 2,** p. 1850 – 1853 (1974).
[12] H. Keller, W. Müller-Beissenhirtz, and E. Neumann, Klin. Wschr. **45,** 314 – 316 (1967).
[13] R. C. Jackson and R. E. Handschuhmacher, Biochemistry **9,** 3585 – 3590 (1970).
[14] A. Arens, E. Rauschenbusch, E. Irion, O. Wagner, Kl. Bauer, and W. Kaufmann, Hoppe-Seyler's Z. Physiol. Chem. **351,** 197 – 212 (1970).
[15] J. J. McTigne and R. L. van Etten, Biochim. Biophys. Acta **523,** 422 – 429 (1978).
[16] T. Maita, K. Morokuma, and G. Matsuda, J. Biochem. **76,** 1351 – 1354, Tokyo 1974.
[17] H. M. Levy, P. D. Leber, and E. M. Ryan, J. Biol. Chem. **238,** 3654 – 3659 (1963).
[18] S. F. Andres and M. Z. Atassi, Biochemistry **12,** 942 – 999 (1973).
[19] H. Makino and Y. Inada, Biochim. Biophys. Acta **295,** 543 – 548 (1973).
[20] R. B. Homer and S. R. Allsopp, Biochim. Biophys. Acta **434,** 100 – 109 (1976).
[21] K. H. Röhm, Thesis, University of Marburg 1972.
[22] U. Menge and L. Jaenicke, Hoppe-Seyler's Z. Physiol. Chem. **355,** 603 – 611 (1974).