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STUDY OF THE ROLE OF ARGININE RESIDUES IN BACTERIAL FORMATE DEHYDROGENASE

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Summary

Modification of 12 arginine residues per molecule of formate dehydrogenase (formate NAD^+ oxidoreductase, EC 1.2 1 2) from the methylotrophic bacterium, *Achromobacter parvulus* I, by 2,3-butanedione results in complete inactivation of the enzyme. Inactivation of the enzyme is reversible and proceeds in two steps via formation of the intermediate enzyme-butanedione complex. Coenzymes but not formate effectively protect formate dehydrogenase from inactivation Complete maintenance of enzyme activity and specific protection of one arginine residue per enzyme subunit are achieved on formation of the binary complex, enzyme-NAD, or the ternary complex, enzyme-NAD-azide. One arginine residue is supposed to be located at the NAD-binding site of the formate dehydrogenase active centre

Introduction

A systematic investigation is being carried out in our laboratory into the structure and reaction mechanism of NAD-dependent formate dehydrogenase (formate : NAD^+ oxidoreductase, EC 1 2 1 2) from the methanol-utilizing bacterium, *Achromobacter parvulus* I. To date, the basic physicochemical and kinetic properties of the enzyme have been studied [1—5]. The role of SH groups and histidine residues in catalysis and substrate binding has been elucidated [6,7].

α-Dicarbonyl compounds such as phenylglyoxal, 2,3-butanedione and 1,2-cyclohexanedione are highly specific reagents used for the modification of arginine residues in proteins [8—10]. The important role of the guanidinium arginine group in the substrate and coenzyme binding has been discovered for a number of NAD(P)-dependent dehydrogenases [11—15].

To elucidate the function of the arginine residues in a formate dehydrogenase molecule we have studied the enzyme inactivation under the action of 2,3-butanedione.

Experimental Procedure

Materials

Formate dehydrogenase was isolated from the methylotrophic bacterium, *A parvulus* I, in the presence of EDTA as the enzyme activity stabilizer [5]. The preparations obtained were homogeneous according to electrophoresis in polyacrylamide gels. The value of formate dehydrogenase specific activity was 9 0 μmol/min per mg.

Freshly distilled 2,3-butanedione solutions (Sigma, U.S.A.) were prepared daily. NAD and NADH (Reanal, Hungary) were used without preliminary purification.

Methods

Enzyme activity was measured in a Reaction Rate Analyzer, model 2086 (LKB, Sweden) in 0.05 mM phosphate buffer, pH 7.0, at 37°C and at NAD and formate concentrations of 1.3 mM and 0.3 M, respectively The formate dehydrogenase concentration was determined at 278 nm on a Hitachi spectrophotometer, model 200-20 (Japan), on the basis of the millimolar extinction coefficient of 127 mM^{-1} cm^{-1} [5] The molecular weight of formate dehydrogenase was considered to be 80 000. Fluorimetric measurements were performed in a Hitachi spectrofluorimeter, model 512 (Japan), at 37°C.

Modification of formate dehydrogenase with butanedione was carried out in 0.035 M borate buffer, pH 8.2, at 25°C. Buffer, protective agents when necessary and 0.01—0.20 ml butanedione solution (0.356 M) were added to 0.25 ml of enzyme solution ($1.35 \cdot 10^{-5}$ M) to a total volume of 1 ml. At certain time intervals, 0.05 ml aliquots were withdrawn and the enzymatic activity was measured

Determination of the total number and of essential SH groups in native and modified formate dehydrogenase was performed with 5,5′-dithiobis(2-nitrobenzoate) as reported earlier [7] Tryptophan was determined spectrophotometrically [16].

Amino acid analysis was carried out as described in Ref 17 on a Hitachi amino acid analyzer (Japan), model KLA-3B The enzyme (total amount, 0.3 mg), inactivated to a certain degree (modification was stopped by the addition of an equal volume of 12 M HCl), was hydrolysed in vacuo in 6 M HCl for 24 h at 110°C. To prevent regeneration of the modified arginine residues, thioglycolic acid was added to the samples in the proportions of 8 μl/ml.

The content of arginine residues was calculated relative to the 68 alanine, 68 leucine and 58 glycine residues found in the formate dehydrogenase molecule [5].

The pH dependence of the apparent inactivation rate constant was computed by a standard procedure on a PDP-8 apparatus (Digital, U.S.A.).

The extent of protection of formate dehydrogenase activity by NAD against the inactivation by butanedione was calculated assuming the following inactiva-

tion mechanism

$$E + \text{butanedione} \xrightarrow{k_0} E^*$$

$$E + NAD \underset{}{\overset{K_N}{\rightleftharpoons}} E\text{-}NAD + \text{butanedione} \xrightarrow{k_1} E^* \quad (1)$$

where k_0 is the apparent inactivation rate constant of the free enzyme, k_1 the apparent inactivation rate constant of the complex enzyme-NAD and K_N the dissociation constant of the complex enzyme-NAD.

The experimentally determined inactivation rate constant of formate dehydrogenase (k) is given by Eqn. 2 [19]

$$k = \frac{k_0 + k_1 \dfrac{[NAD]}{K_N}}{1 + \dfrac{[NAD]}{K_N}} \quad (2)$$

The values of $(k_0 - k_1)$ and K_N were determined graphically using a linear transformation (Eqn 3)

$$\frac{1}{k_0 - k} = \frac{1}{k_0 - k_1} \frac{K_N}{[NAD]} + \frac{1}{k_0 - k_1} \quad (3)$$

Results

In the presence of a 1000-fold molar excess of butanedione, complete formate dehydrogenase inactivation is observed in 8 h. The inactivation process follows pseudo-first-order reaction kinetics up to 25—30% of initial enzyme activity (Fig. 1). The dependence of the apparent enzyme activity rate constant on butanedione concentration is presented in the inset of Fig. 1. The pH value exerts the essential influence on the rate of the enzyme modification, decreasing the half-inactivation period from 12.5 min (pH 8.0) to 1.5 min (pH 9.5) at a 9000-fold molar excess of butanedione. The slope of the dependence of the apparent inactivation rate constant vs. pH is close to unity in the pH range 8.0—8.5 (Fig. 2) The pK value calculated on the basis of the data in Fig. 2 equals 8 9 ± 0.15 at 25°C. The pH dependence of the apparent inactivation rate constant could not be determined from the pK of arginine residues. Free arginine (characterized by the pK of the guanidinium group of greater then 12) easily undergoes modification even at pH 7.5 [11]. The apparent pH profile probably can be explained by the ionization of H_3BO_3 (p$K \approx$ 9 0), since stabilization of the postulated intermediate complex by BO_3^{3-} [11] at alkaline pH should accelerate the modification reaction.

Formate dehydrogenase can be inactivated under the action of butanedione even in the absence of BO_3^{3-}. At an 8000-fold molar excess of the modifying agent, the half-inactivation period of the enzyme is about 150 min (pH 8.2, 30°C). Under the same conditions, BO_3^{3-} at a concentration of 0.035 M essentially increases the inactivation rate and substantially decreases $\tau_{1/2}$ to 4.5 min.

Formate dehydrogenase inactivation under the action of butanedione is

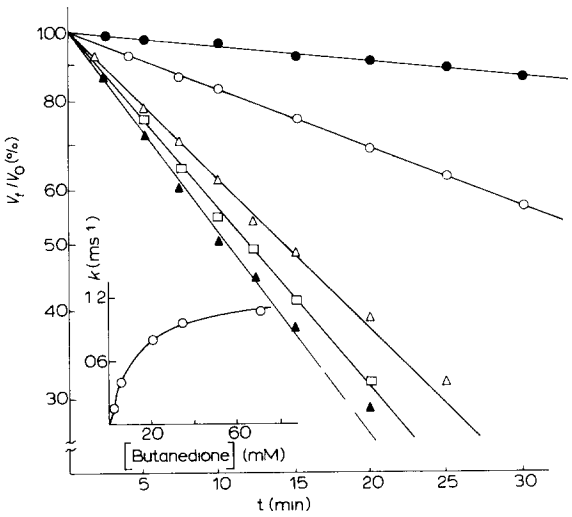


Fig 1 Kinetics of formate dehydrogenase inactivation with butanedione Concentration of modifying agent (mM) 0 69 (•), 2 83 (○), 19 2 (△), 35 6 (□), 71 2 (▲) Enzyme concentration, 3 4 μM, 0 035 M borate buffer, pH 8 2, 25° C (Inset) Dependence of the apparent rate constant of formate dehydrogenase inactivation on butanedione concentration

reversible. The regeneration of the specific activity (up to 80%) of partially inactivated enzyme preparations (20% of the initial activity) is observed just after the removal of both the modifying agent and borate by gel filtration These results are in line with the previously proposed mechanism of modification of the arginine residues with butanedione in the presence of BO_3^{3-} [9,18].

The results of the titration of the total number of SH groups in the native and butanedione-modified formate dehydrogenase (Table I) show that no SH group modification takes place during the enzyme inactivation. The process of enzyme inactivation is correlated with the decrease in the concentration of the essential highly reactive SH groups (Fig. 3)

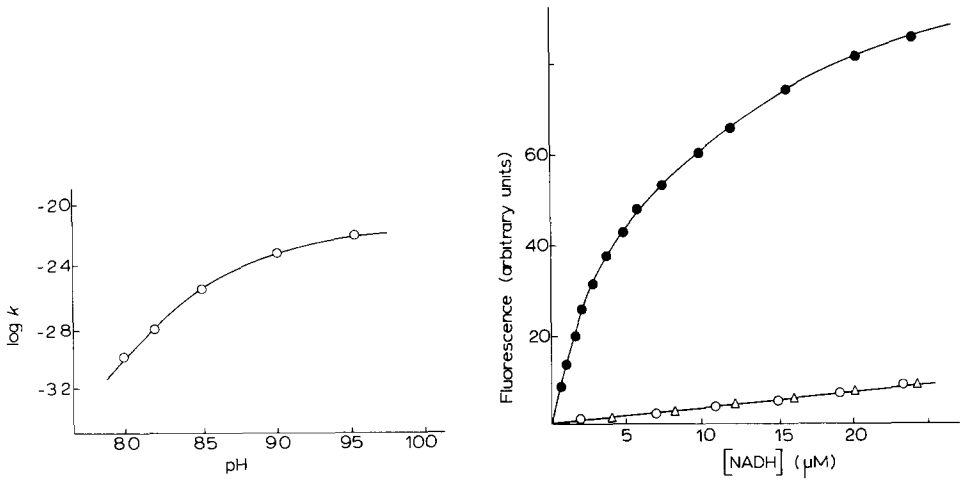
The results of fluorimetric titrations of native and inactivated (3% of initial activity) formate dehydrogenase by NADH are presented in Fig. 4 No enhancement of NADH fluorescence is observed in the presence of inactivated enzyme. The coenzyme also does not quench the protein fluorescence of modified enzyme, i.e., formate dehydrogenase inactivated with butanedione is unable to bind cofactor.

The rate of enzyme inactivation is slowed down by the increase in NAD concentration Table II). Kinetic curves of formate dehydrogenase inactivation under

TABLE I

Titration of SH groups of native and butanedione-modifed formate dehydrogenase (8 M urea in 0 05 M phosphate buffer, pH 7 0, enzyme concentration, 0 404 μM, 5,5′-dithiobis(2-nitrobenzoate), 24 4 μM, butanedione, 1 83 mM)

Degree of enzyme inactivation (%)	0	29 2	46 8	72 7
Number of SH groups per enzyme molecule	12 0	11 9	12 0	12 1



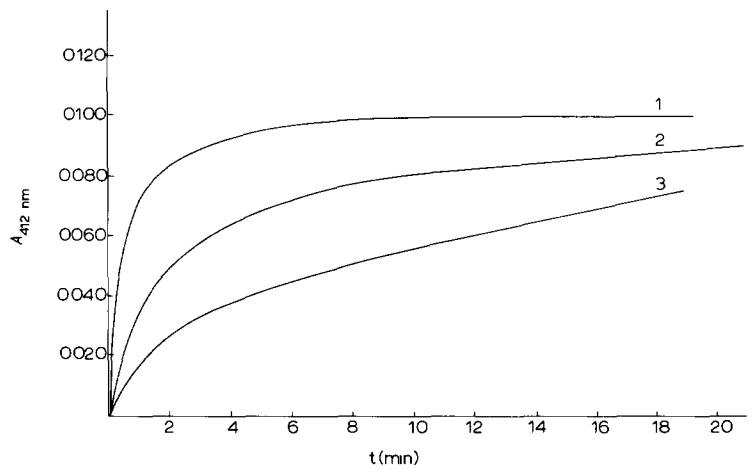


Fig 2 (Top left) pH profile of the apparent inactivation rate constant of formate dehydrogenase Enzyme concentration, 3 4 μM, butanedione concentration, 38 3 mM, 0 035 M borate buffer, pH 8 2, 25°C

Fig 3 (Bottom) Titration of the essential SH groups with 5,5′-dithiobis(2-nitrobenzoate) in native (1) and butanedione-modified (2-60% activity, 3—24% activity, respectively) formate dehydrogenase Enzyme concentration, 3 67 μM, 5,5′-dithiobis(2-nitroberzoate) concentration, 20 mM, 0 035 M borate buffer, pH 8 0, 25°C

Fig 4 (Top right) Enhancement of coenzyme fluorescence by native (●) and butanedione-modified (○) (3% residual activity) formate dehydrogenase △, NADH in the absence of enzyme Formate dehydrogenase concentration, 3 8 μM, 0 035 M borate buffer, pH 8 2, 25°C λ_{ex} = 350 nm, λ_{em} = 450 nm

TABLE II

Dependence of the apparent inactivation rate constant of formate dehydrogenase on NAD concentration (enzyme concentration, 3 4 μM, butanedione, 18 3 mM, 0 035 M borate buffer, pH 8 2, 25°C)

NAD concentration (mM)	0	0 327	0 654	1 31
Inactivation rate constant (s^{-1}) (× 10^4)	7 8	3 73	2 17	1 08

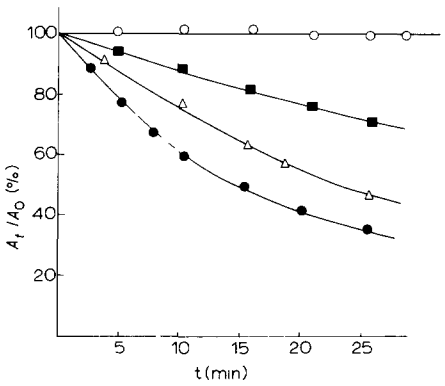


Fig 5 Kinetics of formate dehydrogenase inactivation with butanedione in the presence of protective agents •, control and formate, 0 3 M, △, NADH, 16 5 μM, ■, NAD, 0 65 mM, ○, (NAD + azide), (0 78 + 0 04) mM Enzyme concentration, 3 4 μM, butanedione concentration, 18 3 mM, 0 035 M borate buffer, pH 8 2, 25°C

the action of butanedione in the presence of various compounds are presented in Fig. 5. Coenzymes as well as NAD in the presence of KN_3 effectively protect the enzyme against inactivation. In the presence of NAD + azide, practically complete protection of the enzyme activity during modification is observed.

TABLE III

AMINO ACID CONTENT (RESIDUES PER MOL) OF NATIVE AND MODIFIED FORMATE DEHYDROGENASE

Modified enzyme enzyme concentration, 4 2 μM, butanedione, 6 7 mM, 4 h incubation in 0 035 M borate buffer, pH 8 0, 25°C Protected enzyme the inactivation was performed in the presence of NAD (0 6 mM) and azide (0 36 mM), other conditions as for the modified enzyme

Amino acid	Native enzyme (100% activity)	Modified enzyme 6% activity)	Protected enzyme (100% activity)
Ala	68	68	68
Asp	82	81	81
Glu	58	57	58
Gly	58	59	58
Cys *	12	12	12
His	26	26	26
Ile	28	29	28
Leu	68	67	68
Lys	34	34	34
Met	14	14	14
Phe	16	18	18
Pro	46	46	46
Ser	34	34	35
Thr	44	46	46
Trp **	20	20	20
Tyr	30	31	32
Val	48	48	48
Arg	40	28	30

* Determined by titration with 5,5'-dithiobis(2-nitrogenzoate) in 8 M urea [7]

** Determined spectrophotometrically [16]

Sodium formate does not affect the rate of formate dehydrogenase inactivation

Modification of the enzyme with butanedione is strictly specific. The results presented in Table III indicate that no change in the number of free amino acid residues (except arginine residues) takes place during inactivation. As follows from Table III, up to 12 arginine residues can be modified by butanedione in the absence of protecting agents. At the same time, the number of modified arginine residues in the presence of coenzyme and azide is reduced to 10. Thus, ternary complex formation (enzyme-NAD-azide) affords specific protection of two arginine residues per formate dehydrogenase molecule.

Discussion

Formate dehydrogenase from the methylotrophic bacterium, *A. parvulus* I, like many other NAD-dependent dehydrogenases is inactivated under the action of an excess of butanedione, a reagent which specifically blocks arginine residues in proteins

The pseudo-first-order inactivation kinetics of formate dehydrogenase (Fig. 1) suggest that enzyme inactivation is achieved by the blocking of one arginine residue susceptible to the action of butanedione. In other words, inactivation is associated with the incorporation of a single molecule of the modifying agent per subunit of the formate dehydrogenase dimer. Complete disappearance of enzyme activity during the course of modification testifies to the absence of enzyme forms characterized by a partial loss of catalytic activity.

The hyperbolic character of the concentration dependence of the apparent inactivation rate constant proposes intermediate complex formation between the enzyme and modifying agent prior to inactivation according to the scheme:

$$\text{E} + \text{butanedione} \overset{K}{\rightleftharpoons} \text{E-butanedione} \overset{k}{\rightleftharpoons} \text{E}^*\text{-butanedione} \qquad (4)$$

where K is the dissociation constant of the enzyme-butanedione complex. The linearization of the observed concentration dependence in the double-reciprocal plot makes it possible to evaluate k and K values as 1.3 10^{-3} s^{-1} and 0.010 M, respectively (pH 8.2, 25°C).

The protection studies on formate dehydrogenase in the presence of substrates show that absolute preservation of enzyme activity is achieved on binary and ternary (Fig. 5) complex formation with coenzymes, but not with formate. From the linearization of the data of Table II in a double-reciprocal plot (Fig. 6), values of $(k_0 - k_1)$ and K_N (Eqn. 3) are obtained which equal $7\,9 \cdot 10^{-4}$ s^{-1} and $3.0 \cdot 10^{-4}$ M, respectively. Comparison of the $(k_0 - k_1)$ value with that of k_0 obtained in the absence of protective agents ($7.8 \cdot 10^{-4}$ s^{-1}) shows that the value of k_1 is close to zero.

The ternary complex formation is accompanied by specific protection of one arginine residue per subunit and retention of enzyme activity (Table III). These data together, with the observation that modified formate dehydrogenase does not bind coenzymes, indicates that the arginine residue essential for catalytic activity is situated at the formate dehydrogenase active centre and is located on or near the coenzyme binding site.

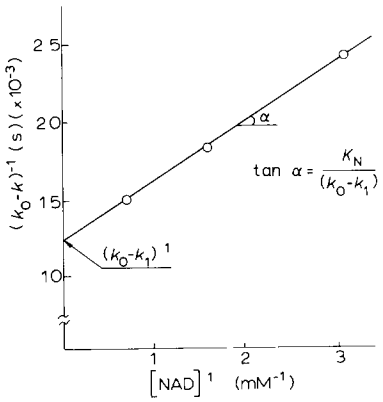


Fig 6 The determination of the extent of protection of the formate dehydrogenase-NAD complex against inactivation with butanedione k_0 and k_1, inactivation rate constants of the free enzyme and enzyme-NAD complex, respectively, k, inactivation rate constant determined experimentally, K_N, dissociation constant of the complex enzyme-NAD

It should be noted that the modification of arginine residues not only inactivates the enzyme, but also leads to the alteration of its native structure A rather sensitive test for the degree of preservation of the native structure of formate dehydrogenase during modification is the reactivity of two essential SH groups of the enzyme towards 5,5'-dithiobis(2-nitrobenzoate). Blocking of arginine residues by butanedione reduces the apparent concentration of the highly reactive SH groups of the enzyme active centre (Fig. 3). At the same time, the total number of formate dehydrogenase thiol groups is preserved during the course of modification (Table I). The same changes in the concentration of the essential SH groups take place during modification of the histidine residues of formate dehydrogenase by diethyl pyrocarbonate [6]. The results obtained show that in the modified formate dehydrogenase, the active centre of the enzyme is a in distorted conformation.

For a number of NAD(P)-dependent dehydrogenases, pyridine nucleotides were shown to protect enzymes from inactivation by arginine-specific reagents [11—15,19]. These results were interpreted by a majority of authors as indicating the participation of one or more arginine residues in coenzyme binding in dehydrogenases. In the case of enzymes such as liver alcohol dehydrogenase and lactate dehydrogenase [20,21], the data of X-ray analysis showed that arginine residues interact with the pyrophosphate coenzyme moiety. Taking into account the extensive homology in the structure and function of the coenzyme-binding domain of NAD(P)-dependent dehydrogenase [22], the essential arginine residues of bacterial formate dehydrogenase are supposed to participate in cofactor binding, interacting with its pyrophosphate moiety.

Thus, we have shown that inactivation of bacterial formate dehydrogenase with butanedione depends on blocking of the single arginine residue located at the active centre of the enzyme. By analogy with other NAD-dependent dehydrogenases, it is supposed that this amino acid residue is located in the coenzyme-binding domain of the active centre and interacts with the pyrophosphate group of the coenzyme.

References

1 Rodionov, Yu V , Avilova, T V , Zakharova, E V , Platonenkova, L S , Egorov, A M and Berezin, I V (1977) Biochemistry (U S S R) 42, 1896—1904
2 Rodionov, Yu V , Avilova, T V. and Popov, V O (1977) Biochemistry (U S S R) 42, 2020—2025
3 Popov, V O , Radionov, Yu V , Egorov, A M and Berezin, I V (1978) Dokl Acad Sci U S S R 239, 214—217
4 Popov, V O and Egorov, A M (1978) Dokl Acad Sci U S S R 240, 221—224
5 Egorov, A M , Avilova, T V , Dikov, M M , Popov, V O , Radionov, Yu V and Berezin, I V (1979) Eur J Biochem 99, 569—576
6 Popov, V O , Radionov, Yu V , Egorov, A M and Berezin, I V. (1978) Biochemistry (U S S R) 43, 1212—1221
7 Popov, V O and Egorov, A M (1979) Biochemistry (U S S R) 44, 207—213
8 Takahashi, K (1968) J Biol Chem 243, 6171—6175
9 Riordan, J F (1973) Biochemistry 12, 3915—3923
10 Blumenthal, K M and Smith, E L (1975) J Biol Chem 250, 6555—6559
11 Lange, L G., Riordan, J F and Vallee, B L (1974) Biochemistry 13, 4361—4370
12 Jornvall, H , Lange, L G , Riordan, J F and Vallee, B L (1977) Biochem Biophys Res Commun 77, 73—76
13 Nagradova, N K and Asryants, R A (1976) FEBS Lett 69, 246—248
14 Levy, H R , Ingulli, J and Afolayan, A (1977) J Biol Chem 252, 3745—3751
15 Riordan, J F (1979) Mol Cell Biochem 26, 71—92
16 Bredderman, P J. (1974) Anal Biochem 61, 298—301
17 Spachman, D H , Stein, W H and Moore, S (1958) Anal Chem 30, 1190—1206
18 Yang, P C and Schwert, C W (1972) Biochemistry 11, 2218—2224
19 Dickenson, C J and Dickinson, F M (1977) Biochem J 161, 73—82
20 Eklund, H , Nordstrom, B , Zeppezauer, E , Soderlund, G , Ohlsson, I , Biowe, T , Soderberg, B -O , Tapia, O , Branden, C -I and Akeson, A (1976) J Mol Biol 102, 27—59
21 Holbrook, J J , Liljas, A J , Steindel, S J and Rossman, M G (1975) Enzymes, Vol II, part A, 3rd edn , pp 191—292, Boer, P , New York
22 Rossman, M G , Liljas, A J , Branden, C -I and Banaszak, C J (1975) Enzymes, Vol II, part A, 3rd edn , pp 61—102, Boer, P , New York