EFFECT OF MODIFICATION OF CERTAIN AMINO ACID RESIDUES ON ENZYME ACTIVITY OF D-3-HYDROXYBUTYRATE DEHYDROGENASE FROM BACTERIUM *Paracoccus denitrificans*

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We examined the effect of several modifying reagents on the activity of the title enzyme. The results show that one histidine residue participates in the interaction of the enzyme with the substrate; one cysteine residue binds near to the nicotine amide moiety of the coenzyme molecule and its role is to induce conformational changes leading to the formation of enzyme aggregates with an increased catalytic power. The enzyme does not contain essential tyrosine and tryptophan residues. The results of the experiments with the modification of additional amino acid residues permit us to make preliminary conclusions only based on the knowledge of the protective effect of the individual ligands: One arginine residue may be involved in the binding of the coenzyme, the residues of lysine and serine may be localized in the substrate binding site.

D-3-Hydroxybutyrate dehydrogenase belongs to NAD-dependent dehydrogenases[1]. The fundamental molecular characteristics of this enzyme and the kinetics of its action have been elucidated[1,2]. The enzyme is a tetramer of relative molecular weight about 130 000 and shows an ordered mechanism with preferential coenzyme binding. The most typical feature of the kinetics of action of this enzyme is hysteresis[1,2]: The course of the enzyme reaction is nonlinear because of the formation of enzyme aggregates showing an increased catalytic power. This aggregation process is initiated by the coenzyme[1,2].

This communication has been designed to provide basic data on the role of various amino acid residues in coenzyme and substrate binding and in aggregation processes. This analysis is based on the results of the examination of the effect of some modifying reagents on the enzyme activity. The protective effect of specific ligands of the enzyme (above all of the coenzyme and of the substrate) was tested in modification reactions, so that the results of these experiments might be interpreted more reliably.

EXPERIMENTAL

D-3-Hydroxybutyrate dehydrogenase was prepared from the bacterium *Paracoccus denitrificans* as described elsewhere[1]. The enzyme concentration is given as molar concentration of enzyme subunits (active centers)[1]. Coenzymes NAD and NADH (grade III) were from Boehringer

(Mannheim, F.R.G.), ADP-ribose and DL-lactic acid from Sigma (St. Louis, MO, U.S.A.) and sodium DL-3-hydroxybutyrate was purchased from BDH Chemicals (Poole, Great Britain). 2,3-Butanedione was a product of Fluka (Buchs, Switzerland), monoiodoacetic acid and *p*-chloromercuribenzoic acid were from Serva (Heidelberg, F.R.G.). Diethyl dicarbonate was purchased from Lachema (Brno, Czechoslovakia). The remaining modifying reagents (phenylmethanesulfonyl fluoride, pyridoxal-5-phosphate, N-bromosuccinimide, and N-acetylimidazole) were from Sigma (St. Louis, MO, U.S.A.), Tris-(hydroxymethyl)-aminomethane (Tris) was a product of Merck (Darmstadt, F.R.G.).

The activity of the enzyme was measured spectrophotometrically at 340 nm in Cary 118 Spectrophotometer (Varian, U.S.A.) in 0·1 mol l^{-1} Tris–HCl buffer at pH 8·5 in the presence of 2 mmol l^{-1} NAD and 20 mmol l^{-1} DL-3-hydroxybutyrate at 25°C. The reactions were triggered by the addition of the enzyme to a final concentration of 10 nmol l^{-1}. In the modification reaction the enzyme (c. 0·5 nmol l^{-1}) was incubated in the presence of the reagent under the conditions specified below. The enzyme activity after various modification times was assayed as described above. The degree of modification by *p*-chloromercuribenzoate and diethyl dicarbonate was determined spectrophotometrically[3,4]. The kinetics of the modification processes was analyzed by plotting semilogarithmically the actual relative residual activity, x, *versus* time using equation $x = (A_t - A_\infty)/(1 - A_\infty)$, where A_t stands for the actual relative enzyme activity determined at time t after the beginning of the modification reaction, A_∞ is the relative enzyme activity for $t \rightarrow \infty$ (*i.e.* at the time where no further activity decrease can be observed); the enzyme activity before the beginning of the modification (*i.e.* for $t = 0$) is regarded as unity.

RESULTS

The effect of several more or less specific modifying reagents on the activity of D-3-hydroxybutyrate dehydrogenase both alone and in the presence of potential protective ligands was assayed. The ligands used were coenzymes (NAD and NADH), a fragment of the coenzyme molecule (ADP-ribose), a mixture of the coenzyme with a competitive inhibitor and the substrate (*i.e.* DL-lactate)[2] and in some cases the substrate (DL-3-hydroxybutyrate) alone. The basic results of experiments with the modification of cysteine, serine, arginine, lysine, histidine, tyrosine, and tryptophan residues are given in Table I. When less specific modifying reagents were used the reaction conditions were chosen so that possibly amino acid residues of one type only might react. The effect of monoiodoacetate and acetic anhydride (not shown in Table I) was analogous to the effect of *p*-chloromercuribenzoate and pyridoxal phosphate, respectively. Since the effect of *p*-chloromercuribenzoate and of diethyl dicarbonate on the activity of the enzyme appeared most interesting the kinetics of modification of the enzyme by these two reagents was examined in more detail (Figs 1 and 2). The additions of 5 mmol l^{-1} NAD or of an identical quantity of NAD and 10 mmol l^{-1} lactate had an effect analogous to that of 0·5 mmol l^{-1} NADH (Fig. 1). The addition of a mixture of 5 mmol l^{-1} NAD and 10 mmol l^{-1} lactate had a similar effect as the addition of 20 mmol l^{-1} 3-hydroxybutyrate (Fig. 2). In experiments with the free enzyme the activity after complete modification by *p*--chloromercuribenzoate under the conditions described was close to 50% of its

TABLE I

Modification of D-3-hydroxybutyrate dehydrogenase by reagents specific for certain amino acid residues. The enzyme (0·5 μmol l^{-1}) was incubated with the reagents under the conditions described; unless specified the reaction was carried out in 0·1 mol l^{-1} sodium phosphate, pH 7, 25°C. The residual activities are means of 3—4 measurements and are expressed in per cent of the control (the enzyme incubated in the absence of the reagent)

Reagent (concentration)	Modified residue (conditions)	Residual activity (%) in presence of protective ligands				
		—	5 mmol l^{-1} NAD	0·5 mmol l^{-1} NADH	3 mmol l^{-1} ADP-ribose	5 mmol l^{-1} NAD + 10 mmol l^{-1} lactate
p-Chloromercuribenzoate[5] (0·1 mmol l^{-1})	Cys (20 min)	50	95	95	60	95
Phenylmethanesulfonyl fluoride[6] (0·3 mmol l^{-1})	Ser (20 min, pH 7·5)	25	30	30	35	80
2,3-Butanedione[6] (10 mmol l^{-1})	Arg (50 min, 0·05M borate)	45	50	55	50	85
Pyridoxal phosphate[7] (0·2 mmol l^{-1})	Lys (60 min)	55	80	85	80	85
Diethyl dicarbonate[4] (0·2 mmol l^{-1})	His (15 min, pH 6·5)	30	15	45	40	55
N-Bromosuccinimide[8] (0·1 mmol l^{-1})	Trp (40 min)	60	60	60	60	65
N-Acetylimidazole[9] (0·1 mmol l^{-1})	Tyr (80 min)	80	85	85	85	85

original value ($A_\infty \approx 0{\cdot}5$) and even when higher concentrations of the reagent were used or when the reaction period was prolonged no additional marked activity decrease was observed. In contrast, the reaction of diethyl dicarbonate with the free enzyme (even in mixture with NAD and ADP-ribose) led to a practically complete loss of enzyme activity ($A_\infty \to 0$). The degree of modification of sulfhydryl groups and of histidine residues (for the observed activity decrease to c. 50% or to zero, respectively) as determined in repeated experiments with the free enzyme corresponded to 1·0–1·7 reacted residues in both cases. These data are subject to a considerable experimental error. A suppression of the hysteretic effects after the modification of the free enzyme by *p*-chloromercuribenzoate was observed.

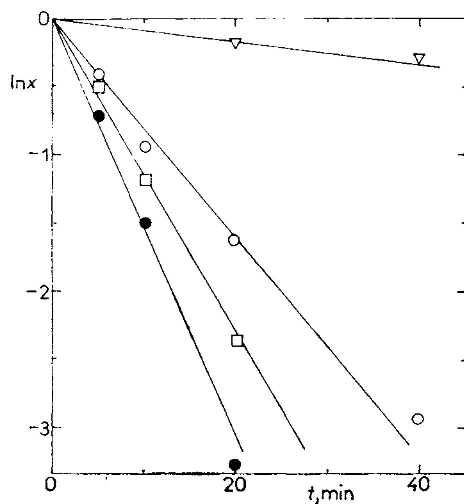

FIG. 1

Semilogarithmic plot of residual relative enzyme activity, x, *versus* time (min) of incubation with *p*-chloromercuribenzoate. The enzyme 0·5 µmol l^{-1} was incubated with 0·1 mmol l^{-1} reagent in 0·1 mol l^{-1} sodium phosphate buffer, pH 7, $x = (A_t - A_\infty)/(1 - A_\infty)$, (*cf.* Experimental), $A = 0{\cdot}5$, ● enzyme alone, □ enzyme in presence of 20 mmol l^{-1} DL-3-hydroxybutyrate, ○ enzyme in presence of 3 mmol l^{-1} ADP-ribose, △ enzyme in presence of 0·5 mmol l^{-1} NADH

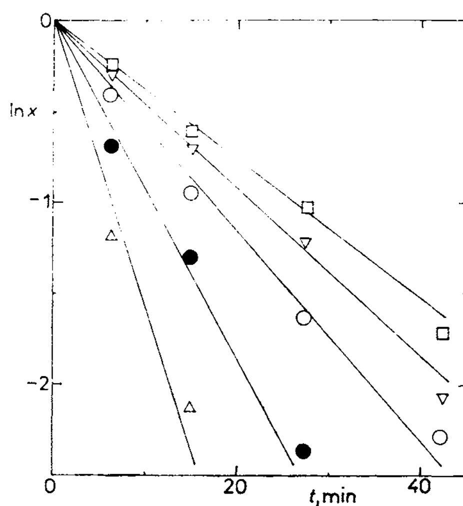

FIG. 2

Semilogarithmic plot of residual relative enzyme activity, x, *versus* time (min) of incubation with diethyl dicarbonate. The enzyme (0·5 µmol l^{-1}) was incubated with 0·2 mmol l^{-1} reagent in 0·1 mol l^{-1} sodium phosphate buffer, pH 6·5. The symbols used are the same as in Fig. 1, Δ addition of 5 mmol l^{-1} NAD, $A_\infty = 0$

DISCUSSION

The results presented in Table I and Figs 1 and 2 indicate a different degree of importance of the amino acid residues tested for the enzyme activity and also their different localization in the enzyme molecule. Preliminary conclusions only can be deduced from the data obtained (with the exception of those on the modification of histidine and cysteine). As obvious from Table I, the tyrosine and tryptophan residues are not localized in the active center of the enzyme and are not of essential character. The data show that the role of the lysine residues is more important: the protective effect of the coenzyme and of its fragment (ADP-ribose) indicates that this residue could be involved in the binding of the diphosphate moiety of the enzyme. The stabilization of the coenzyme in the binding site through electrostatic interactions with the positively charged group of the enzyme (a lysine or arginine residue) has been demonstrated with all NAD-dependent dehydrogenases which have been studied in more detail[10,11]. In contrast, it is likely that the arginine and serine residues will participate in the binding of the substrate (*cf.* data in Table I and the pronounced protective effect of 20 mmol l^{-1} 3-hydroxybutyrate in both cases (not shown in Table I)). The positive effect of arginine may serve electrostatic stabilization of the substrate binding (like in the case of lactate, malate, and hydroxybutyrate dehydrogenase from mammalian tissues, *cf.*[10–12]); the serine residue may form a hydrogen bond between the molecule of the substrate and the binding site of the enzyme. A more exact consideration of the role of lysine, arginine, and serine residues, however, would require additional experiments.

A very important role in the reaction catalyzed by *Paracoccus denitrificans* D-3-hydroxybutyrate dehydrogenase play cysteine and histidine residues (Table I, Figs 1 and 2). The linear character of the plots shown in Fig. 2 and the degree of modification determined indicate that one histidine residue is localized in the substrate binding site and is necessary for the enzyme reaction. The effect of diethyl dicarbonate is suppressed more markedly in the presence of substrate or ternary enzyme–NAD–lactate complex (Table I, Fig. 2). A fact deserving interest is that the presence of NAD considerably enhances the rate of modification of this residue. This may be due to the proximity of the positively charged nicotine amide ring of NAD which will then speed up by its electrostatic effect the course of the reaction of histidine with the modifying reagent. This finding shows at the same time that the histidine residue is not essential for the coenzyme binding even though it is localized near to its active moiety. The presence of the histidine residue in the neighborhood of the bound substrate has been demonstrated with numerous NAD-dependent dehydrogenases[10,11,13].

The role of the cysteine residue in the active center of the enzyme is also important yet slightly different. The modification of this residue does not lead to a complete loss of enzyme activity; a marked protective effect has been observed in the presence

of both coenzyme forms only (Table I, Fig. 1). These findings are in accordance with the imagination that the cysteine residue is not essential for substrate binding and for the course of the reaction catalyzed by the enzyme yet is localized very close to the nicotine amide moiety of the coenzyme. The interaction of this residue with the bound coenzyme is most likely responsible for the initiation of the transition of the enzyme from the nonaggregated (less active) form to the aggregated form. This is evidenced both by a decrease of enzyme activity after modification of this residue to c. 50% (similarly the activity of the nonaggregated form corresponds to c. 50% of activity of the aggregated form[2]) and also by the observation that the hysteretic effects are suppressed in the enzyme which was modified by *p*-chloromercuribenzoate.

REFERENCES

1. Matysková I., Kovář J., Racek P.: Biochim. Biophys. Acta *839*, 300 (1985).
2. Kovář J., Matysková I.: Biochim. Biophys. Acta *871*, 302 (1986).
3. Benesch R., Benesch R. E.: Methods Biochem. Anal. *10*, 43 (1962).
4. Wallis R. B., Holbrook J. J.: Biochem. J. *133*, 183 (1973).
5. Bergmayer H. U., Gawehn K., Klotzsch H.: Biochem. J. *102*, 423 (1967).
6. Whitaker J. R., Perez-Villasenor J.: Arch. Biochem. Biophys. *124*, 70 (1968).
7. Anderson B. M., Anderson G. D., Churchick J. E.: Biochemistry *5*, 2893 (1966).
8. Friesheim J. H., Huennekens F. M.: Biochemistry *8*, 2271 (1969).
9. Riordan J. F., Wacker W. E. C., Vallee B. J.: Biochemistry *4*, 1758 (1965).
10. Holbrook J. J., Liljas A., Steindel S. J., Rossmann M. G. in the book: *The Enzymes* (P. D. Boyer, Ed.), 3rd ed. Vol. 11, p. 191. Academic Press, New York 1975.
11. Banaszak L. J., Bradshaw R. A. in the book: *The Enzymes* (P. D. Boyer, Ed.), 3rd ed., Vol. 11, p. 360. Academic Press, New York 1975.
12. Kebhaj M. S. E., Latruffe N., Gaudemer Y.: Biochim. Biophys. Res. Commun. *96*, 1569 (1980).
13. Brändén C. I., Jörnvall H., Eklund H., Furugren B. in the book: *The Enzymes* (P. D. Boyer, Ed.), 3rd ed., Vol. 11, p. 103. Academic Press, New York 1975.

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