THE AMINO ACID SUBSTITUTION TYROSINE 88 → HISTIDINE IN THE LYSOZYME OF PHAGE T4: THE MOLECULAR CAUSE OF THE COLD-SENSITIVE PHENOTYPE OF THE MUTANT *cseBU56*

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Received 1 July 1974

1. Introduction

The temperature behaviour of enzyme reactions is of great influence on the cardinal temperatures of microbial growth [1]. We have been interested in the biochemical causes of temperature sensitivity of cold-sensitive (*cs*–) mutants. Changes of the temperature requirements can often be related to altered enzymes [2].

We decided to study the relation of *cs*-mutations in the lysozyme gene (*e*-gene) to temperature requirements of phage T4D. The phage T4D was mutagenized with 5-bromouracil. Cold-sensitive mutants with a mutation in the *e*-gene were selected. After purification of the lysozyme of the wild type and of a selected mutant we searched for an amino acid substitution within the mutant lysozyme by peptide mapping.

2. Methods

Fig. 1 summarizes the experimental steps. The mutagenesis, the selection, the purification procedure of the enzymes, the performance of the disc electrophoresis, the method of estimating the efficiency of substrate binding as well as the peptide mapping and the spectroscopic estimation of the tryptophan and tyrosine content are desribed elsewhere [3–5]. In the chloroform test the selection was based on the absence of a halo, which indicates a reduced lysozyme activity around and in a plaque. The cold sensitivity was defined by the efficiency of plating (e.o.p.) at 20°C as compared with 37°C and with the wild type behaviour. The *cs*-mutants in the *e*-gene were characterized by combined biological and enzymatic tests.

3. Results

After mutagenesis 15 halo less-mutants were selected by the chloroform test. Seven of these mutants showed a decreased e.o.p. at 20°C. Table 1 demonstrates the

Table 1
Efficiency of plating of the mutant *cseBU56* before and after shift-up as compared with the wild type T4D

	Temperature (°C)	% e.o.p.
Wild type	37	100
	20	62
	16	21
Mutant	37	100
	20	0.01
after shift-up		78

The Petri dishes were incubated for 30 hr at the indicated temperatures, in the shift-up experiment 20 hr at 20°C and 48 hr at 37°C. E.o.p. was defined in all cases at 37°C as 100%.

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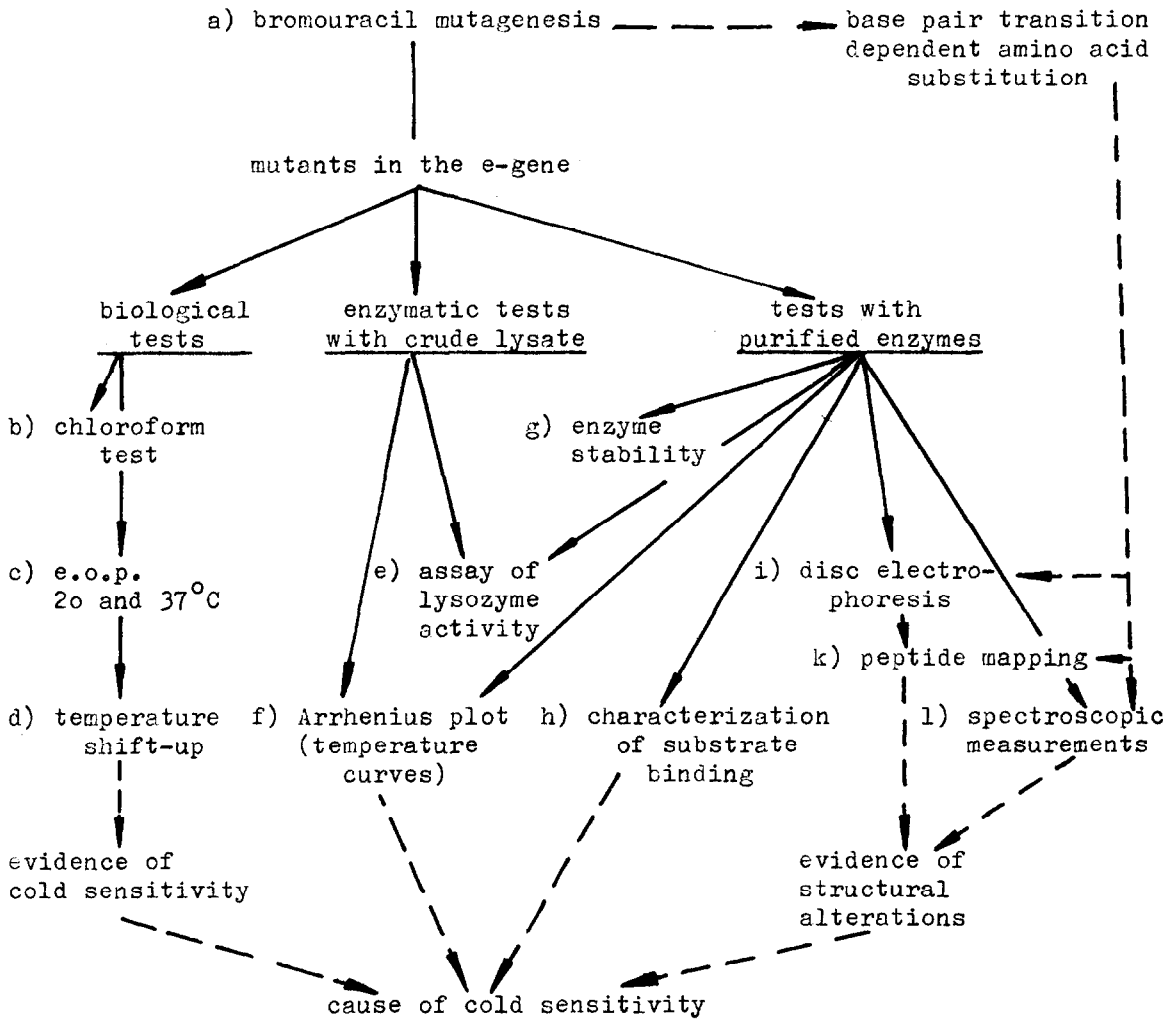

Fig. 1. Strategy of analysis of the cold sensitivity of mutants in the lysozyme gene of phage T4. ———— experiments; – – – – conclusions.

e.o.p. of one representative before and after temperature shift-up. The fast reversibility of the plaque forming ability suggests that a cold-sensitive biosynthesis [6] of the enzyme responsible can be excluded.

In order to achieve a more specific selection of mutants in the *e*-gene an analysis of the temperature curves of the lysozyme reaction was carried out. In the Arrhenius plot for all mutants and the wild type a linear dependence appeared between the logarithm of the velocity of reaction and the reciprocal of absolute temperature in the range from 14°C to 35°C. From the slope of the curves the activation energy was calculated. Four out of of the seven *cs*-mutants revealed a changed activation energy. The most pronounced difference to the wild type was found with the mutant *cseBU56* (table 2). The slope of the curve and the activation energy of the lysozyme reaction of the mutant was significantly increased. In crude lysates the lysozyme activity as measured at 25°C amounted to only 10% of that of the wild type. The enzyme stabilities at 4°C did not differ.

The enzymes of the wild type and of the mutant *cseBU56* were purified according to Tsugita et al. [7]. The purification was judged by disc electrophoresis. The preparation of the wild type enzyme was pure and

Table 2
Activation energies of the lysozyme reaction with an *E. coli* cell wall preparation before and after purification of the enzymes

	Activation energy (kcal/mole)	
	Wild type	Mutant *cseBU56*
Crude lysate	11.1 ± 0.8	17.4 ± 2.1
Purified enzymes	11.5 ± 0.7	15.9 ± 1.0

Data including standard deviation.

represented one protein species. The preparation of the mutant enzyme showed two protein species in a proportion of 1:1. The yield of protein was only 70% of that of the wild type lysozyme. Both mutant proteins could be separated by an additional ion exchange chromatography on carboxymethyl-cellulose columns. Only one mutant protein showed lysozyme activity; it was named 'active mutant lysozyme'. The other protein was the 'inactive protein'. In order to find possible charge differences between the wild type and the active mutant lysozyme both enzymes were investigated by disc electrophoresis in urea in one gel at pH 4.5. The active mutant lysozyme had a higher anodal mobility suggesting an increased number of cationic charges.

An amino acid substitution was demonstrated on the peptide maps by thin layer chromatography and high voltage electrophoresis after tryptic digestion of the enzymes. In fig. 2 the peptide map of the wild type is shown. Theoretically 23 tryptic peptides and 3 free arginine residues, i.e. 24 single spots, had been expected [8]. After ninhydrin staining 27 spots were found, presumably caused by incomplete tryptic digestion. The peptides were further characterized by staining for tyrosine residues and by fluorescence spectroscopy on thin layers for tryptophan residues. The identification of the tyrosine and tryptophan-containing peptides was based on the nomenclature proposed by Inouye et al. [8]. The six tyrosine residues were distributed to 5 peptides (one of which, Tp5, contained 2 tyrosine residues), the three tryptophan residues to 3 peptides. All other peptides were arbitrarily indexed.

After ninhydrin staining the peptide map (fig. 3) of both the active mutant lysozyme and the inactive protein was in good accord with the map in fig. 2. There was no evidence for additional proteins deviating from

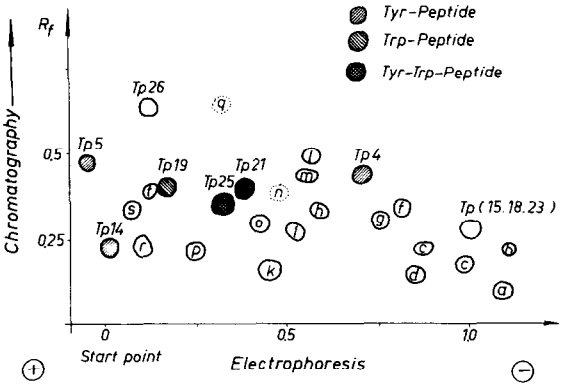

Fig. 2. Peptide map of the T4D wild type lysozyme (tryptic digestion). Arginine served as a marker for the electrophoretic migration distance of the peptides.

the primary structure of the phage lysozyme. However, the tyrosine staining demonstrated only 4 as compared with the 5 tyrosine peptides of the wild type enzyme. The tyrosine peptide Tp14, which contains tyrosine 88, was missing. However, a new spot was localized near peptide Tp19. The new peptide had a higher electrophoretic mobility than peptide Tp14 at pH 3.7 indicating an increased number of cationic charges. Other minor deviations were discussed elsewhere [5]. The decreased number of the tyrosine residues was verified by comparison of the protein spectra in guanidine hydrochloride at different pH values [9]. The molar proportion of tryptophan to tyrosine was 3.0–4.8 for both mutant

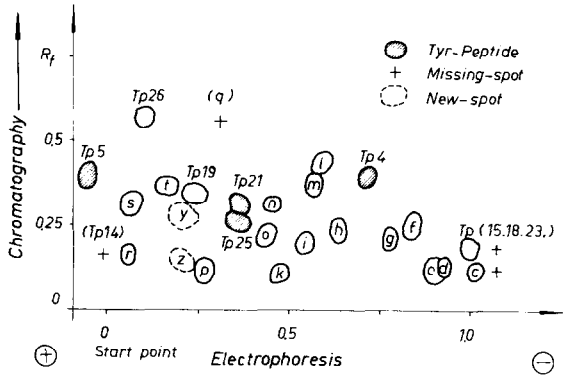

Fig. 3. Peptide map of the mutant *cseBU56* lysozyme (tryptic digestion).

proteins. The estimation of the amount of tyrosine at alkaline pH yielded also 5 residues only.

Taking into account the mechanism of bromouracil mutagenesis, which allows base pair transitions as one kind of point mutation [10], only two amino acid substitutions were possible: tyrosine → histidine or tyrosine → cysteine. The higher cationic charge of both the active mutant enzyme and the altered peptide Tp14 indicated the substitution of tyrosine by histidine.

In addition to the modified temperature behaviour (table 2) this amino acid substitution caused several functional alterations of the enzyme. These referred to its specific activity, its substrate binding, and its stability. The specific activity of the active mutant lysozyme amounted to 40% (125 U/E_{280}) of that of the wild type enzyme. In fig. 4 differences in the binding efficiency of the enzymes to lyophilized cell walls of *E. coli* are shown. At low enzyme concentrations significantly less active mutant enzyme as compared with that of the wild type was bound. The weakening of the substrate binding could contribute to the decreased specific activity, because the activity assay is performed at these low enzyme concentrations. The lower stability of the mutant lysozyme was evident by heat, acid denaturation and on an Amberlite IRC50 column. On it the mutant enzyme was reversibly inactivated. An additional effect was the stronger inhibition by unidentified acid inhibitors present in the cell wall preparation.

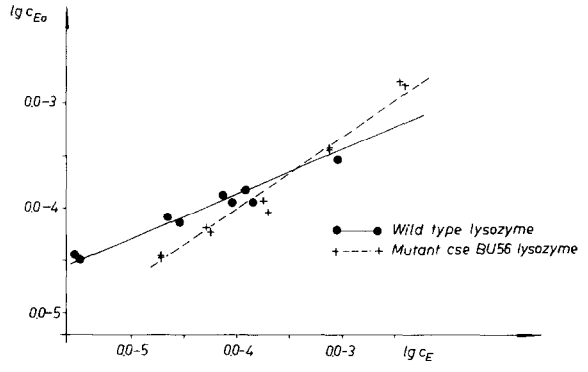

Fig. 4. Lysozyme binding to *E. coli* cell walls at 0°C. c_{Ea} adsorbed lysozyme in mg, c_E lysozyme in the concentration of equilibrium. The experiments were analysed according to the adsorption equation of Freundlich suggested in ref. [11].

4. Discussion

Cold sensitivity of microorganisms induced by mutations are frequently caused by altered enzyme functions [2]. Generally two cases can be distinguished: a decrease of the enzyme activity [12] over the whole temperature range investigated, without a change of activation energy, or a selectively stronger decrease of the enzyme activity of the mutant by lowering the tempe rature as compared with the wild type enzyme. For the second case some examples exist. From the data given by several authors [13–15] we calculated an increase of the activation energy of the enzyme reactions of those *cs*-mutants. Only Yura [16] has pointed out a possible relationship between the cold-sensitive phenotype and an increased activation energy of an enzyme reaction. Our results clearly demonstrate such a relationship. Considering the yield of total enzyme, the proportion of active and inactive mutant protein after enzyme purification, the decreased specific activity, and the increased activation energy, only 4% lysozyme activity, which is not sufficient for lysis of bacteria, exists at 20°C in the mutant enzyme as compared with 100% of the wild type activity at 37°C. Without the change of activation energy a level of 12% at 20°C is calculated. This level of activity may be sufficient for the essential function of lysis of the infected bacteria at 20°C [17]. But the increase of the activation energy of the reaction caused the activity at 20°C to fall below a critical level. The results suggest that the activation energy of essential enzyme reactions could play a key role for the minimal growth temperature.

The results also reflect the significance of tyrosine 88 for the phage lysozyme function. The substitution is situated in a region where up to now substitutions by mutations were not known [18]. The lability of the active mutant lysozyme suggests a possible role of tyrosine 88 for the native protein conformation. The data of both the altered specific activity and the weakening of substrate binding point to an effect on the substrate binding properties. These changes may be the basis for the higher activation energy of the mutant enzyme reaction.

References

[1] Babel, W., Rosenthal, H. A. and Rapoport, S., Acta Biol. Med. Germ. 28, 565.

[2] Ingraham, J. L. (1966) Cryobiology 6, 188.
[3] Liebscher, D. H., Dauberschmidt, R. and Rosenthal, H. A. (1974) Z. Allg. Mikrobiol. 14 (5).
[4] Dauberschmidt, R., Liebscher, D. H., Thiele, B. J., Rapoport, S. M., Heitmann, P. and Rosenthal, H. A. (1974) Acta Biol. Med. Germ. 32, 315.
[5] Liebscher, D. H., Dauberschmidt, R., Rapoport, S. M., Heitmann, P. and Rosenthal, H. A. (1974) Mol. Gen. Genet., in press.
[6] Condon, S. and Ingraham, J. L. (1967) J. Bacteriol. 94, 1970.
[7] Tsugita, A., Inouye, M., Terzaghi, E. and Streisinger, G. (1968) J. Biol. Chem. 243, 391.
[8] Inouye, M., Okada, Y. and Tsugita, A. (1970) J. Biol. Chem. 245, 3439.
[9] Edelhoch, H. (1967) Biochemistry 6, 1948.
[10] Freese, E. (1963) in: Molecular genetics (Taylor, H. J., ed.), Vol. 1, pp 207, Academic Press, New York & London.
[11] McLaren, A. D. and Packer, L. (1970) Advan. Enzymol. 33, 245.
[12] O'Donovan, G. A. and Ingraham, J. L. (1965) Proc. Natl. Acad. Sci. U.S. 54, 451.
[13] Abd-el-al, A. and Ingraham, J. L. (1969) J. Biol. Chem. 244, 4033.
[14] Abd-el-al, A. and Ingraham, J. L. (1969) J. Biol. Chem. 244, 4039.
[15] Hoffmann, B. and Ingraham, J. L. (1970) Biochim. Biophys. Acta 201, 300.
[16] Yura, T. (1959) Proc. Natl. Acad. Sci. U.S. 45, 197.
[17] Mark, K. K. and Chen, I. (1972) Biochem. Biophys. Res. Commun. 46, 1102.
[18] Okada, Y., Amagase, S. and Tsugita, A. (1970) J. Mol. Biol. 54, 219.