An Enzyme Degrading Reduced Nicotinamide–Adenine Dinucleotide in *Proteus vulgaris*

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(Received 3 May 1978)

Cell-free extracts of a strain of *Proteus vulgaris* degrade NADH to reduced nicotinamide riboside, adenosine and two molecules of phosphate. The system is weakly active in fresh cell extracts, but activity is increased about 10-fold on rapid heating to 70–100°C. On returning to room temperature, the activity returns rapidly to its initial low value but can be re-activated by again heating to 70–100°C. Reversible activation can also be effected by extremes of pH or by treatment with 8M-urea. Activation appears to be due to reversible changes in conformation of the protein of the enzyme rather than to combination of the enzyme with a heat-labile inhibitor. The active form can be stabilized by addition of PP_i. The system, which also possesses 5'-nucleotidase activity not separable from the NADH pyrophosphatase, requires Co²⁺ (0.4mM) for maximum activity. Although activated at relatively high temperatures, it is not enzymically active until cooled to 50–60°C. It may be purified by affinity chromatography (with NAD⁺ as ligand) to an activity over 400 times that of the crude cell extract, and yields only one major band on polyacrylamide-gel electrophoresis.

In studies in this Department on the oxidation of NADH, the rapid decline in A_{340} after addition of cell extracts of *Proteus vulgaris* to NADH ceased when the A_{340} was still 20–30% of the original value. (Similarly, when NADH was incubated anaerobically with *Proteus* extracts and oxaloacetate added later, the malate dehydrogenase reaction did not cause complete loss of A_{340} .) Extracts of several other bacteria, as expected, oxidized NADH completely under similar conditions (J. D. McGarry, personal communication). Preliminary studies showed that the *Proteus* strains investigated degraded NADH enzymically to reduced nicotinamide riboside, adenine and P_i. The A_{340} of reduced nicotinamide riboside is identical with that of NADH, but the substance is not oxidized by NAD⁺-linked dehydrogenases. NAD⁺- or NADH-hydrolysing systems had been reported in *Proteus vulgaris* (Swartz *et al.*, 1956, 1958) and in *Staphylococcus aureus* and *Staphylococcus albus* (Swartz & Merselis, 1962). Unusual features of these systems included a substantial stimulation of activity on addition of Co²⁺ at the surprisingly high concentration of 5mM, and a remarkable activation of the enzyme through heating at 100°C; this they attributed to a combination of heat-stable enzyme and a thermolabile inhibitor. Swartz *et al.* (1958) found that NAD⁺-splitting activity (EC 3.6.1.9) was accompanied by a 5'-nucleotidase activity (EC 3.1.3.5). They did not

isolate either the enzyme or the thermolabile inhibitor as separate entities. Neu (1967*a,b*, 1968) reported both Co²⁺ activation and a thermolabile inhibitor effect when investigating 5'-nucleotidases from a number of bacteria; the enzyme from *Proteus* also showed a low extent of NADH hydrolysis. Mather & Knight (1969) have reported in *Pseudomonas fluorescens* an enzyme destroying NAD⁺, heat-stable but accompanied by a heat-labile inhibitor. There was no accompanying 5'-nucleotidase, and the products were nicotinamide ribotide and AMP. Our *Proteus* system shared some of the properties of the enzyme described by Swartz *et al.* (1958), but showed significant differences that merited further investigation: in particular, study of the relation between the thermostable enzyme and the reported thermolabile inhibitor.

Materials and Methods

Growth of organism and enzyme preparations

Proteus vulgaris L. 10 (from the Department of Biochemistry, University of Liverpool) was grown on medium G-Y containing (per litre): KH₂PO₄, 3g; Na₂HPO₄, 7g; (NH₄)₂SO₄, 1g; NaCl, 1g; acid-hydrolysed casein (Oxo Ltd., London S.E.1, U.K.), 2g; Bactopeptone (Oxo), 1g; yeast extract (Difco Laboratories, Detroit, MI, U.S.A.), 5g; monosodium

L-glutamate, 2g; pH 7.0. The yeast extract contributed approx. 1.4mg of nicotinamide/litre. The medium (500ml in 1-litre conical flasks), sterilized at 10^5 Pa for 20min, was inoculated with 1 ml of a broth culture and incubated on a rotary shaker at 250 rev./min at 30°C for 12h (late exponential phase). The cells were harvested by centrifugation (23 000g, 15 min), washed twice with 50mM-potassium phosphate/sodium phosphate, pH 7.0, then resuspended in 1.8ml of this buffer/g wet wt. The cells were disrupted by passing twice, at 4°C, through a French pressure cell at 4×10^7 Pa, centrifuged at 4°C for 1½h at 130 000g and the resulting supernatant was used as the crude enzyme preparation, either immediately or after storage at 4°C.

Heat-activated enzyme preparations. Conditions for activation of the enzyme are discussed in the Results section, but unless otherwise stated activation was accomplished by heating at 73°C for 2min in the presence of 10mM-sodium pyrophosphate and, where appropriate, removal of denatured insoluble protein by centrifugation at 38 000g for 15 min at 4°C.

Enzyme and other assays

Unit. An enzyme unit is the amount that transformed 1 μ mol of substrate in 1 min at the experimental temperature (normally 37°C). The activity of a preparation is the number of enzyme units present in the volume containing 1 g of protein before heating or other activating treatment. (The protein content at the time of assay may be somewhat less, owing to loss by denaturation during heat-activation.)

Buffers. Enzyme activity was measured at pH 7.4 in 50mM-Tris/HCl buffer unless stated otherwise.

NADH pyrophosphatase. Assay, control and blank tubes each contained, in 3ml of buffer, 1.2 μ mol of CoCl_2 and 16.5 units of malate dehydrogenase (EC 1.1.1.37; pig heart; B.C.L., Lewes, E. Sussex BN7, U.K.). The assay and control tubes, which contained also 0.9 μ mol of NADH, were equilibrated at 37°C for 4min, then the enzyme preparation (in 0.1 ml of buffer) was added to the assay tube, and both tubes were incubated for a further 10min to allow hydrolysis of the NADH in the assay tube. Oxaloacetate (5.3 μ mol in 0.1 ml of buffer) was then added to all three tubes, and assay and control tubes were incubated for 1 min at 37°C. In the control tube, this completely oxidized the NADH originally added to NAD^+ . In the assay tube, only the NADH that had survived hydrolysis by the pyrophosphatase would be oxidized. The reduced nicotinamide riboside, whose A_{340} is identical with that of NADH, would survive. After addition of 0.1 ml of the enzyme preparation to the control and blank tubes, A_{340} in the assay and control tubes was read against the blank; the difference between A_{340} in assay and control tubes gave a measure of the absorption corresponding to degraded NADH. Degradation of

NADH was linear with respect to time for up to 30% removal of the NADH added and over a period of at least 20min.

With preparations containing NADH oxidase activity, anaerobic cuvettes filled with O_2 -free N_2 were used. This applied mainly to crude cell extracts, since the activation of the NADH pyrophosphatase by heating destroyed the NADH oxidase.

NADH oxidase. The assay cuvette contained 0.45 μ mol of NADH in 2.9 ml of buffer and the reference cuvette 2.9 ml of buffer. Both cuvettes were placed in the spectrophotometer at 25°C for 2.5 min and the enzyme preparation (0.1 ml) was added. The rate of fall in A_{340} was measured.

Malate dehydrogenase. This was assayed by the rate of fall in A_{340} on incubation of the enzyme preparation with oxaloacetate and NADH at 25°C and pH 7.4 (50mM-Tris/HCl). The preparations used were examined for NADH oxidase activity, but none contained sufficient to interfere with the malate dehydrogenase assay.

5'-Nucleotidase. For this assay, 1.9 ml of buffer containing 0.6 μ mol of AMP and 0.8 μ mol of CoCl_2 was equilibrated for 4 min at 37°C. A sample (0.1 ml) of a suitable dilution of the enzyme preparation was added and incubated for 10 min at 37°C, then 0.1 ml of 10% (w/v) trichloroacetic acid was added and the liberated P_i measured (Fiske & SubbaRow, 1925). The control (enzyme added immediately before the trichloroacetic acid) was subtracted.

Protein. This was determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Material for affinity chromatography. Sepharose 4B (Pharmacia, Uppsala, Sweden), with NAD^+ attached through a C_6 spacer arm provided by ϵ -aminohexanoic acid, was activated by the method of Cuatrecasas (1970); the remainder of the preparation was carried out as described by Larsson & Mosbach (1971).

Results

Loss of activity in crude cell extracts

A crude enzyme preparation, activity 130 units/g of protein, decreased to 40 units after 1 h at 18°C or overnight at 4°C. The initial activity of a sample thus depended to some degree on how rapidly the disintegration and centrifugation procedures were performed. Unless otherwise stated all crude cell extracts were kept (either at room temperature or at 4°C) for long enough for the activity to fall to a minimum but stable value.

Activation of the enzyme system

Activation of the enzyme by heating. A remarkable feature of the enzyme was its activation on heating

for 30s at 98°C or 2min at 73°C. The latter conditions were used in all heat-activation experiments (unless stated otherwise). At 98°C destruction of the enzyme occurred within a few minutes and accurate control of heating time was difficult. At 73°C there was no significant gain or loss if heating was prolonged to 3min, and the NADH oxidase present in the cell extracts was inactivated at this temperature, thus avoiding the need to conduct the enzyme assays in anaerobic cuvettes. The concentration of protein during heating was critical; about 0.65mg/ml proved best. After heating (except where immediate assay was required), denatured protein was removed by centrifugation (38000g, 15min, 4°C) and the supernatant used as the enzyme preparation. The heat-activated enzyme was stable only in the presence of PP_i . Otherwise, like the freshly prepared extracts, it lost activity rapidly. When an untreated preparation (activity 25 units/g of protein) was heated (73°C, 2min) the activity increased to 110 units, but declined to 30 units on standing overnight at 4°C. Another sample from the same preparation was similarly heated in the presence of 10mM- PP_i and gave an activity of 240 units falling only to 220 units after 72h at 4°C. No significant loss occurred after 4h at 18°C. These results were not affected by the addition of 0.4mM- Co^{2+} before heating. PP_i thus appeared to stabilize the heated enzyme; 10mM was the optimum concentration. The following experiments showed that its action was easily reversed.

(1) A crude enzyme preparation was heated with 10mM- PP_i , yielding an activity of 240 units/g of proteins. After dialysis (30mM-Tris/HCl, pH 7.4, 3 days, 4°C), activity was 25 units. PP_i (10mM) was added and activity re-assayed (25 units). The preparation was now re-heated (73°C, 2min) and an activity of 170 units was obtained.

(2) A second preparation (activity 270 units/g of protein; vol. 2ml), obtained by heating in the presence of 10mM- PP_i , was passed down a column (15cm $\times$ 1.5cm) of Sephadex G-25 at 4°C. The PP_i passed through in the void volume and the enzyme fraction was then collected; an activity of 155 units was recovered. After incubation for 30min at 29°C, this decreased to 50 units and remained at this value on incubation for a further 30min. PP_i (10mM) was now added and the preparation heated (73°C, 2min); the activity was 230 units.

These experiments showed that the cycle of heat activation and inactivation on standing (in the absence of PP_i) could be repeated. The explanation of Swartz *et al.* (1958) of the heat activation in terms of a heat-labile inhibitor separating from a heat-stable enzyme was not therefore valid in this case.

Heating with 10mM-ATP or AMP yielded 10 and 60% respectively of the activity obtained on heating with PP_i . NAD^+ or NADH (10mM) showed only slight activity.

Activation by urea. A crude enzyme preparation (activity 55 units/g) had an activity of 280 units/g of protein after heating (73°C, 2min, 10mM- PP_i). Another sample was treated with 8M-urea with 10mM- PP_i in 30mM-Tris/HCl, pH 7.4, for 10min at 4°C. Activity was now 110 units, or 40% of the value obtained by heating. The urea was now removed by dialysis against 30mM-Tris/HCl, pH 7.4, containing 10mM- PP_i for 37h at 4°C. Activity changed only slightly (105 units). The PP_i was now removed by further dialysis against 30mM-Tris/HCl, pH 7.4, for 24h at 4°C. Activity decreased to 33 units. After heating in the presence of 10mM- PP_i (73°C, 2min), activity increased to 175 units.

Activation by sonication. A crude enzyme preparation (activity 24 units) yielded 235 units/g of protein after heating (73°C, 2min, 10mM- PP_i). Sonication of another sample (3×30 s, 4°C, in a 500W 20kHz disintegrator, MSE, Crawley, Surrey, U.K.) gave an activity of 32 units. If the same sonication procedure was used in the presence of 10mM- PP_i activity was 80 units.

Products of reaction. Swartz *et al.* (1958) reported that the products of reaction were adenosine, nicotinamide riboside and 2P_i . The following confirmatory observations were made: (i) when crude extract was incubated with NADH, for every mol of NADH rendered unavailable for oxidation, 1.8mol of P_i was liberated; (ii) when the products were chromatographed on paper with ethanol and 1M-ammonium acetate (5:2, v/v) (Takei *et al.*, 1966) a powerfully u.v.-absorbing spot was detected at R_F 0.47, the position taken up by authentic adenosine; on elution this showed the u.v.-absorption spectrum characteristic of adenosine.

Requirement for metal ions. Addition of Co^{2+} was necessary for maximum activity. The optimum concentration was difficult to define because of the interaction of Co^{2+} with PP_i . In the absence of PP_i maximal effect was obtained with 0.1mM- Co^{2+} ; under normal assay conditions (10mM- PP_i) the optimum Co^{2+} concentration was 0.4mM. A preparation activated by heating (73°C, 2min, 10mM- PP_i) had an activity of 20 units/g of protein without addition of any metal ion (apart from the Na^+ accompanying the PP_i). Addition of metal ions (0.4mM) gave activities as indicated: Co^{2+} , 250 units; Ni^{2+} , 165 units; Mn^{2+} , 155 units; Mg^{2+} , 70 units; Cu^{2+} , Zn^{2+} , Ca^{2+} gave values approximately equal to the 20 units obtained without metal ion addition, but Cr^{2+} , Ba^{2+} , Sn^{2+} , Al^{3+} and Fe^{2+} were inhibitory.

Co^{2+} appeared to enter into a firm combination since little loss of activity occurred on prolonged dialysis against 30mM-Tris/HCl, pH 7.4, containing 10mM- PP_i , with or without 1mM-EDTA. Enzyme activated by treatment at pH 2.35 showed the same requirement for Co^{2+} and the same firm combination as the heat-activated preparations.

Influence of growth conditions on enzyme activity. Activity remained approximately constant from mid-exponential phase through to the stationary phase of growth. The typical activity of 250 units/g of protein in the heated preparations grown on the medium as described in the Materials and Methods section decreased to 170 units/g when yeast extract was replaced by an equivalent concentration (10 μ M) of nicotinamide; this was about 10 times the nicotinamide concentration required by the organism for optimum growth. Addition of 1 mM-AMP to the growth medium (containing 10 μ M-nicotinamide) raised activity to 550 units; 1 mM-NAD⁺ raised it to 780 units. AMP is a substrate for the 5'-nucleotidase activity associated with the NADH pyrophosphatase.

Nature of the activation process. Swartz *et al.* (1958) explained the increase in activity of their NADH pyrophosphatase on heating as being due to the inactivation of a thermolabile inhibitor disclosing the full activity of a heat-stable enzyme. This cannot apply to the present system, since when the crude extract was heated in the absence of PP_i (or heated in the presence of PP_i and the latter removed by dialysis) the enzyme reverted to its low-activity state, but was re-activated by reheating as described above. The data so far could have two explanations. (1) An enzyme and an inhibitor, both heat-stable, which separated on heating, but which (in the absence of PP_i) gradually recombined at lower temperatures. (2) An enzyme that can exist in either a fully active form or a low-activity form, heat causing a reversible conformational change to the fully active form, and reversion to the low-activity form being inhibited by PP_i.

Separation of enzyme and inhibitor

Attempts were therefore made, by using Sephadex chromatography and affinity chromatography, to separate an inhibitor from the enzyme.

Sephadex chromatography. Cell extract was activated by heating in the presence of buffer and 10 mM-PP_i and the insoluble protein was removed by centrifugation (38000g, 15 min). The supernatant (9 ml) was loaded on a column (45 cm $\times$ 2.5 cm, V_0 75 ml) of Sephadex G-200 (Pharmacia) and eluted (buffer + 10 mM-PP_i) in 5 ml fractions. The enzyme emerged as a single symmetrical peak. The most-active fraction (V_e 130 ml) represented approx. 24-fold purification and the V_e/V_0 ratio corresponded to a mol.wt. of approx. 92000 (from data of Andrews, 1965, assuming a globular shape). Total recovery of activity was 127%, suggesting that some inhibitory material had been removed. Swartz *et al.* (1958) similarly reported a slight increase in activity at some stages of purification of their enzyme. Most of the activity was in nine fractions collected from the column. These were individually passed down a

column (15 cm $\times$ 1.5 cm) of Sephadex G-25 to remove the PP_i present and incubated for 1 h at 29°C. The activity in all fractions fell to a low value, representing a fairly constant fraction of the activity present before the Sephadex G-25 treatment. This result could be explained only either if heat-activation was a reversible conformational change or by dissociation of an inhibitor whose chromatographic behaviour on Sephadex G-200 was identical with that of the enzyme itself.

Affinity chromatography. This technique offered a very specific method for determining whether the heat-activation was due to conformational change in a single enzyme species, or to dissociation of an inhibitor. A Sepharose-NAD column would be expected to retain the NADH pyrophosphatase enzyme, but not any inhibitor, unless the latter, also, combined with NAD⁺ or NADH.

Sepharose-NAD⁺ was prepared as described in the Materials and Methods section and its specificity checked as follows. (1) Neither bovine serum albumin nor lactate dehydrogenase was retained by Sepharose without the NAD⁺ ligand. (2) Bovine serum albumin was not retained by Sepharose-NAD⁺. Lactate dehydrogenase was retained on a Sepharose-NAD⁺ column and eluted by buffer containing its specific coenzyme NADH (0.15 mM). Heat-activated NADH pyrophosphatase preparation was retained on the Sepharose-NAD⁺ column and eluted only by buffer containing 0.15 mM-NADH. Although Co²⁺ was required for maximum activity of the enzyme, retention by Sepharose-NAD and elution by NADH occurred irrespective of whether Co²⁺ was present or not. The enzyme did not bond to Sepharose-NAD⁺, however, in the presence of 10 mM-PP_i. Crude preparations show pyrophosphatase activity, so PP_i may be a substrate for the enzyme and thus prevent attachment to NADH. For these experiments the enzyme was heat-activated in the absence of PP_i and the chromatography and enzyme assays of the fractions were performed as rapidly as possible.

Enzyme (0.5 unit; 0.94 mg of protein in 3 ml) obtained by heating a preparation (in 30 mM-Tris/HCl, pH 7.4, without PP_i) was applied immediately to a column (10 cm $\times$ 0.6 cm) of Sepharose-NAD⁺ followed by 12 ml of 'plain' buffer (30 mM-Tris/HCl, pH 7.4); no enzyme was eluted. Buffer containing 0.15 mM-NADH was now added and 3 ml fractions were collected. Chromatography was carried out at 4°C and a flow rate of 1 ml/5 min. Almost all the activity was in the first 3 ml fraction; this had an activity of 0.15 unit; it fell to 0.06 unit after dialysis (30 mM-Tris/HCl, pH 7.4, 4 h, 4°C), but increased to 0.44 unit on heating in the presence of 10 mM-PP_i. If the inhibitor had been detached from the enzyme on heating, then (except in the unlikely event of its bonding to the Sepharose-NAD⁺ in a manner similar to that of the enzyme itself) it should have been eluted

by the 'plain' buffer. In that case, the enzyme, eluted with NADH, would have exhibited full activity. The inhibitor thus behaved exactly like the enzyme in respect of attachment to, and specific elution from, the Sepharose-NAD⁺ column, and so must be considered as a part of the enzyme itself. Affinity chromatography was also applied to the problem of the low activity present in fresh cell extracts that survived relatively long storage. Was this due to a separate enzyme, or did the inactivation process stop short of completion? Affinity chromatography of crude (unheated) preparations yielded anomalous results, possibly because of other NADH dehydrogenases in the cell extract saturating the ligand. So a crude extract was heat-activated, dialysed (30mM-Tris/HCl, pH 7.4, 4h) and loaded on to the Sepharose-NAD⁺ column. Each fraction was assayed immediately and also after activation by heating; the ratio of activity in the crude extract to that developed after heating was constant throughout. There was no evidence to suggest that the residual activity surviving storage was a different entity from the heat-activated enzyme.

Purification of the enzyme

Affinity chromatography provided highly-purified samples of the enzyme though only in small amounts. A crude preparation (100ml containing 63mg of protein) was activated by heating with PP_i, yielding 16.5 enzyme units. Denatured protein was removed by centrifugation (38000g, 15min, 4°C); 27.5ml of 1% protamine sulphate (in 30mM-Tris/HCl buffer, pH 7.4) was added after stirring for 30min at 4°C and the precipitate was centrifuged (38000g, 15min, 4°C). Then 150μmol of CoCl₂ in 12.5ml of water was added and after standing for 19h at 4°C the preparation was centrifuged (38000g, 15min, 4°C) and the supernatant dialysed against 2 × 2 litres of 30mM-Tris/HCl buffer, pH 7.4 (6h and 15h at 4°C). The volume was now 132ml and contained 31mg of protein and 20.6 units of enzyme. This represents rather more activity than the starting material, but it had been noted on several occasions that treatment with protamine sulphate caused some rise in activity.

Of the above material, 54ml (8.4 units of enzyme and 12.7mg of protein) was loaded in two 27ml batches on to the Sepharose-NAD⁺ column described in the previous section, and, after washing with 30mM-Tris/HCl buffer, pH 7.4, the column was eluted with the same buffer containing 0.05mM-NADH (4 × 3ml fractions collected), 0.10mM-NADH (3 × 3ml fractions) and 0.15mM-NADH (2 × 3ml fractions). Some activity was obtained in each of the 0.05mM-NADH fractions, but about 50% was recovered in the first of the 0.10mM-NADH eluates. Total recovery was 6.7 units in 0.058mg of protein, representing a specific activity of 118

units/mg compared with 0.26 and 0.66 unit/mg respectively for the starting material and the preparation before affinity chromatography. A 450-fold overall purification had been achieved and 81% of the activity applied to the column was recovered. On polyacrylamide-gel disc electrophoresis (Zweig & Whitaker, 1967), only one major band of activity was seen, but five faint minor bands were detectable.

5'-Nucleotidase activity

Since AMP is split rather faster than NADH, only free phosphate, rather than nucleotides, can be detected as products of the action of the enzyme on NADH. Activation by heat and the requirement of PP_i for stability are identical for the two activities. The 5'-nucleotidase has a somewhat lower pH optimum (pH 6.5) and the enhancement of activity by added Co²⁺ occurs only below pH 7.5. 5'-Nucleotidase activity was determined at each stage of purification; the ratio of activities towards AMP and to NADH was 3.76 for the starting material and 3.63 for the purified preparation; none of the fractions obtained gave ratios deviating significantly from these values.

Discussion

Heat-activated enzymes degrading NAD⁺ have been reported from a number of bacterial sources; that described by Swartz *et al.* (1958) showed the closest resemblance to the system now described. Even allowing for the different strains of *Proteus* used and the different conditions of growth and enzyme assay, important distinctions remained. The enzyme of Swartz *et al.* (1958), once activated (by heating at 100°C in the presence of PP_i), apparently retained its activity; they interpreted their results in terms of a thermostable enzyme being released from the cell along with a thermolabile inhibitor, and claimed that these two could be at least partially separated as individual entities. Our system showed a low activity even without heat activation; if heat-activated, its activity gradually declined on standing at room temperature (or 4°C) to about the pre-heating value, but could be re-activated almost fully on re-heating. Our system thus behaved as a single entity, which was converted into an active form at high temperatures or extreme pH, but reverted to the inactive form under normal conditions. The unitary concept was borne out by our failure to separate enzyme and inhibitor functions by heating at various temperatures, by dialysis after heating or by chromatography on either Sephadex or an affinity-chromatography system. The latter is particularly noteworthy, since this would fail to differentiate between an enzyme and inhibitor only if they both combined with the substrate (NAD⁺) with the same degree of affinity. Heating thus does not remove an inhibitor;

rather, it brings about conformational changes that render the enzyme active, and the stabilizing function of PP_i must be to assist in holding the enzyme in this configuration. The activation/inactivation changes do not appear to affect the substrate-binding group directly, since the 'inactive' form of the enzyme is bound to, and eluted from, Sepharose- NAD^+ in a manner identical with the active form.

Although the enzyme shows considerable stability at high temperatures, it is active only below about 50°C (cf. Swartz *et al.*, 1958). Presumably, on cooling from high temperatures, two conformational changes occur: a rapid change, at about 50°C , giving rise to an active enzyme, and a much slower reversion to the inactive (or relatively inactive) form in which the system is present before heating.

R. D. thanks the Medical Research Council for a studentship which enabled this study to be carried out.

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