Structure of *Neurospora* Malate Dehydrogenase. I. Reconstitution from Acid and Urea*

Kenneth D. Munkres

ABSTRACT: Conditions for the reconstitution of *Neurospora* malate dehydrogenase after acid and urea denaturation are described. The results of these experiments, together with genetical and physicochemical evidence presented elsewhere, indicate that the structure of this enzyme is of the tetrameric form $\alpha\alpha\alpha\beta$, where α and β are polypeptides of equal molecular weight and nonidentical amino acid sequence. The proposed mechanism of reversible denaturation by acid at pH 2.8 involves the dissociation of the tetramer to a trimeric form of α and β monomer, with the reassociation being directed by specific noncovalent interactions intrinsic to the amino acid sequence of the polypeptide chains.

The cooperative interaction and folding of the four polypeptide chains is apparently essential for the formation of the single active site. Physiological and genetical implications of these results are discussed.

In a previous paper on the genetic alteration of *Neurospora* malate dehydrogenase,[1] we proposed, on the basis of observations of amino acid replacement and comparative sedimentation constants and from genetic theory, that the quaternary structure of this enzyme is of the tetrameric form $\alpha\alpha\alpha\beta$ (Munkres and Richards, 1965a). The results of additional experiments reported here and in the accompanying paper indicate that the structure of this enzyme is indeed as previously proposed. The present paper describes processes of reconstitution of active enzyme after acid and urea denaturation.

Materials and Methods

Special chemicals used were: NADH,[2] Grade III, 98% (Sigma Chemical Co.), 2-mercaptoethanol (Eastman Kodak Co.), bovine serum albumin, fraction V (Armour), and Sephadex (Pharmacia Fine Chemicals Inc.). All other chemicals were analytical reagent grade. Urea solutions were freshly prepared, passed through a mixed-bed resin (Bio-Rad AG118), and adjusted to pH 8.2 with trimethylamine to minimize cyanate concentration (Stark *et al.*, 1960).

Methods of purification and assay of MDH and protein determination were previously described (Munkres and Richards, 1965b). Dissociation and reassociation experiments were carried out in a constant temperature bath at 20 ± 0.05° unless otherwise noted. Measurements of pH were made to the nearest 0.01 unit with glass electrodes and a Radiometer, Type PHM4b, pH meter.

The conditions employed for the reconstitution of MDH from acid solution are essentially those described briefly by Deal *et al.* (1963) who reported the reversible dissociation of several enzymes other than MDH. The general experimental protocol is as follows (specific deviations from this procedure are noted in figures and tables of the text). Purified, lyophilized, salt-free MDH is dissolved to a concentration of 0.025 to 0.05% in a buffer containing: 10 mM sodium citrate, 50 mM NaCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol at a final pH of 6.8. This buffer is denoted as buffer A. The enzyme solution is dialyzed against buffer A at 4° for 24 hr in 0.6 cm acetylated dialysis tubing. Dialysis tubing was acetylated by the method of Craig (1960). The exact amount of 1 M citric acid required to lower the pH of buffer A to 2.8 and the amount of 1 N NaOH required to back-titrate to pH 6.8 are determined for each batch of solutions. To 50 μl of dialyzed enzyme solution equilibrated at 20°, *ca.* 35 μl of 1 M citric acid is added. The acidic solution and a control solution, prepared by the addition of 35 μl of 1 M NaCl to enzyme solutions at pH 6.8, are incubated at 20°. Samples (10 μl) of the acidic and control solutions are removed at given intervals and diluted with 50 mM sodium phosphate buffer, pH 7.0 (containing 0.1% BSA), in an ice bath. The remainder of the acidic solution is neutralized to pH 6.8 with *ca.* 87 μl of 1 N NaOH. Sodium chloride (87 μl of a 1 M aqueous solution) is added to the control solution. Reneutralized and control solutions are incubated at 20° for reconstitution of activity. Samples of 10 μl are removed at given intervals and diluted as described above. Assays of diluted solutions are carried out immediately after the above procedure was completed.

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[1] E.C.1.1.1.37. L-malate:NAD oxidoreductase.

[2] Abbreviations: NADH, reduced nicotinamide–adenine dinucleotide; BSA, bovine serum albumin; MDH, malate dehydrogenase.

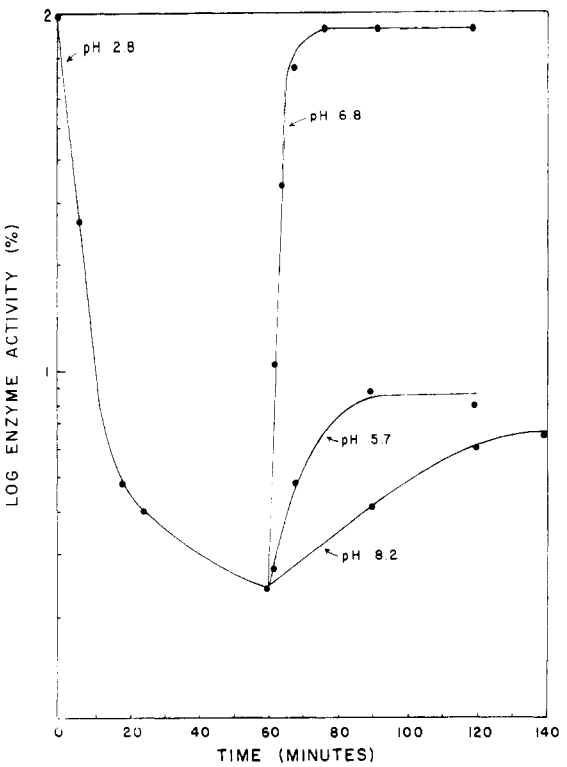

FIGURE 1: Kinetics of the reconstitution of enzymic activity at different pH. The ordinate gives the total yield (in logarithm of per cent of original MDH activity) and the abscissa gives the time. MDH at 0.2 mg/ml in buffer A was dissociated by the addition of 1 M citric acid to pH 2.8, and reconstitution was effected by adjusting the pH of the solutions to the values indicated in the figure. The protein concentrations during reconstitution were 0.165 mg/ml (3 μM). The initial specific activity remained constant during the experiment in controls similarly diluted and maintained at pH 6.8. All solutions were at 20 $\pm$ 0.05°.

Dilute enzyme solutions used for the determination of sedimentation constants were concentrated at 4° by several batch extractions with dry Sephadex G-25. Ultracentrifugal analyses were carried out with a Spinco Model E ultracentrifuge with schlieren optics at 59,780 rpm and 20°. Measurements on the photographic plates were made with a Mann two-dimensional microcomparator.

Results

Reconstitution of MDH after Acid Denaturation. Acidification of solutions of *Neurospora* MDH to pH 2.8 at 20° leads to loss of enzyme activity, the half-life being 4 to 6 min (Figure 1). Control solutions at pH 6.8 retained constant activity for at least 4 hr at 20°. No protein precipitation is observed at pH 2.8 at protein concentrations up to 3 mg/ml. Reneutralization of the acidic solution within 1 to 2 hr after acidification leads to a rapid and, under optimum conditions, complete

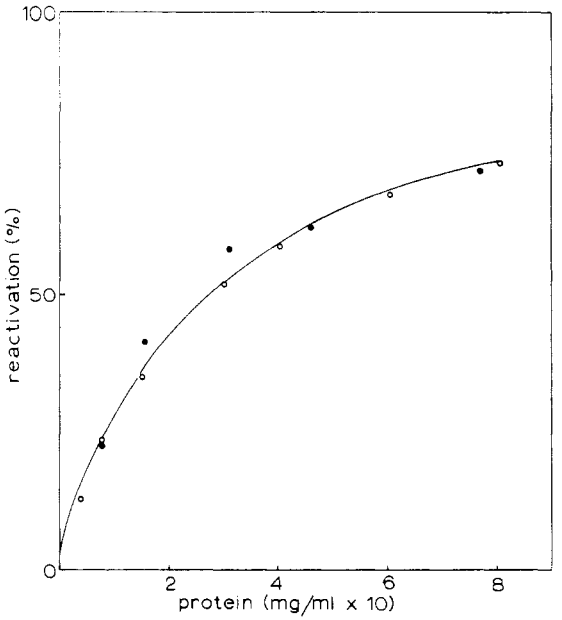

FIGURE 2: Reconstitution of MDH after acid denaturation as a function of protein concentration. Experimental conditions are described in the legend of Figure 1 and in the text under Methods. Reconstitution incubations were at pH 6.8 for 60 min at 20°. O—O, calculated ($K_A = 6 \times 10^5$); ●—●, observed.

reconstitution of activity with a half-life from 4 to 6 min (Figure 1). The optimum pH for reconstitution is near neutrality. The relative initial apparent first-order rates of reconstitution at pH 8.2, 6.8, and 5.7 are 15, 240, and 80 mμmoles $l.^{-1}$ min^{-1}, respectively, at a protein concentration of 3 μM (Figure 1).

The extent of reconstitution at pH 6.8 after 60-min incubation, which is effectively infinite time, as a function of the initial protein concentration is plotted in Figure 2. An apparent association constant of 6×10^5 is estimated from these results where the reaction is of the type A + B → C and the reactants are present in equimolar concentrations. There is some variation in the extent of reconstitution observed with a given protein concentration with different preparations and in different experiments (for example, compare Figures 1 and 2). This variation may possibly reflect the initial specific activity of the enzyme preparation and/or the extent of reduction of sulfhydryl groups (see below). At protein concentrations above 1 mg/ml, the reconstitution process is inversely proportional to concentration, with less than 10% reactivation at 3 mg/ml. The kinetics of reconstitution in the concentration range of 1–3 mg/ml could not be fitted to any reaction of fourth order or less.

Measurements of reconstitution in the temperature range 2 to 20°, at pH 6.8 and a protein concentration of 3 μM, indicate that the process is a linear function of temperature. An Arrhenius activation energy of 13.6 kcal was obtained.

The effect of mercaptoethanol on the reconstitution

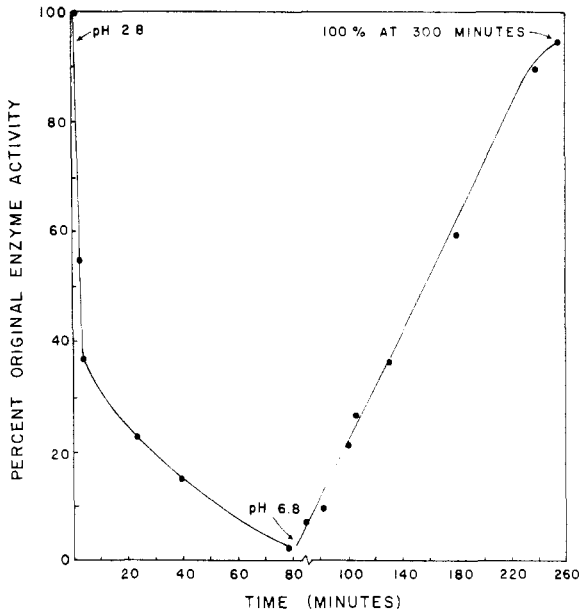

FIGURE 3: Reconstitution of MDH from cell extracts after acid denaturation. *Neurospora* mycelial acetone powder (Munkres and Richards, 1965b) was extracted for 30 min at 4° in buffer A and, after centrifugation, was dialyzed against buffer A for 6 hr at 4°. The extract contained 7.7 mg of protein/ml and MDH with specific activity of 7.1. The extract was acidified and reneutralized as described in the legend of Figure 1 and in the text under Methods. The final concentration of MDH during reconstitution was 12.4 μg/ml (0.23 μM).

process is summarized in Table I. Enzyme that is either freshly dissolved in buffer A (which contains 1 mM mercaptoethanol) or dialyzed 24 hr against buffer A exhibits about the same specific activity, and full reconstitution of activity is obtained after acid denaturation (treatments A and B, Table I). However, if either the mercaptoethanol is entirely omitted (treatment D) or is removed from the enzyme solution by rapid passage through a Sephadex G-25 column and omitted in the reconstitution system (treatment C), the initial specific activity is 33–50% of that obtained in the complete system and only 11–12% reconstitution is obtained. The functional role of EDTA or NaCl in the reconstitution system is apparently minor or *nil* compared to that of mercaptoethanol (treatments E and F).

The reconstitution process also obtains in cell-free extracts of *Neurospora*. In Figure 3, the half-time of reconstitution from a cell extract is 90 min. Similarly, the half-time of reconstitution of pure MDH at a concentration near that of the crude enzyme is 80 min. These results indicate that the process is equally efficient in either crude cell extracts or with pure enzyme. [The MDH concentration in crude extracts is determined by employing the apparent turnover number in the standard assay of 2100 μmoles of NADH min^{-1} (mg protein)$^{-1}$ (Munkres and Richards, 1965b) and the observed specific activities.]

TABLE I: Effect of Mercaptoethanol on Reconstitution of MDH.

Treatment[a]	Solvent[b]	Initial Sp. Act.[c]	Reconstitution (%)[d]
A	Complete	2,120	100
B	Complete	1,860	100
C	Complete minus mercaptoethanol	880	12
D	Complete minus mercaptoethanol	620	11
E	Complete minus mercaptoethanol and EDTA	460	7
F	Complete minus mercaptoethanol, EDTA, and NaCl	590	8

[a] Treatment conditions: A, freshly dissolved MDH in buffer A; B, dialyzed 24 hr at 4° in buffer A; C, removed mercaptoethanol from treatment B by passage through a column of Sephadex G-25 and elution with buffer A minus mercaptoethanol; D, freshly dissolved MDH in buffer A minus mercaptoethanol; E, same as D minus EDTA; F, same as E minus NaCl. [b] The complete system contained buffer A (10 mM sodium citrate, 1 mM mercaptoethanol, 1 mM EDTA, 50 mM NaCl, pH 6.8) and protein. [c] μmoles NADH oxidized min^{-1} (mg protein)$^{-1}$ at 25°. [d] Reconstitution is expressed as the percentage of the initial specific activity recovered after acidification at pH 2.8 for 60 min. The protein concentration during reconstitution was 0.15 mg/ml. Temperature 20°.

Direct evidence for reversible dissociation of MDH after acid treatment is obtained by examining the sedimentation constants (Table II). The sedimentation constant of the native enzyme at pH 6.8 is 4.57. After incubating the enzyme 1 hr at 20°, pH 2.8, the constant is 3.11; however, the schlieren pattern of these solutions

TABLE II: Sedimentation Constants of Native, Dissociated, and Reconstituted Malate Dehydrogenase.

Preparation	Buffer[a]	pH	$s_{20,w}$[b]
Native MDH	P_i	7.0	4.69
Native MDH	Citrate	6.8	4.57
Acidified MDH	Citrate	2.8	3.11[c]
Reconstituted MDH	P_i	7.0	4.67

[a] P_i, 50 mM sodium phosphate; citrate, buffer A (see Materials in text). [b] At protein concentrations of 3 mg/ml. [c] Inhomogeneous (see Results in text).

indicates that the protein is inhomogeneous and contains slower sedimenting material. After gel filtration of the acidified solution at pH 2.8, two protein components are obtained, in a weight ratio of 3:1, with sedimentation constants of 3.65 and 1.81 and molecular weights of 40,500 and 13,500 (Munkres, 1965). Reconstituted MDH at neutral pH yields a single sedimenting component in the ultracentrifuge with a sedimentation constant of 4.67, a value that is identical with the sedimentation constant of the native enzyme. Thus, the reconstitution of activity appears to be accompanied by a reassociation of the polypeptide subunits.

Reconstitution of MDH after Urea Denaturation. The results of preliminary experiments on the reconstitution of MDH activity from urea denaturation by dilution and dialysis are given in Table III. When the urea concentration after denaturation is rapidly reduced by dilution, up to 10% of the original activity is recovered. A slow removal of urea by dialysis in the cold leads to 27% recovery of the original activity. With reference to a dialyzed control sample in buffer at pH 6.8, which unaccountably lost activity (Table III, treatment 3i), the enzyme sample after dialysis from urea was equally as active (Table III, treatment 3ii).

When the enzyme is heated at 100° at pH 6.8, a flocculent, copious, and inactive precipitate is formed (Table IV). The precipitate redissolves instantaneously when the solution is adjusted to 8 M urea. Slow removal of the urea by dialysis in the cold leads to full reconstitution of activity. The significance of the difference in extent of reconstitution in this experiment and a previous and similar one (Table III, treatment 3) is not known.

TABLE III: Reconstitution of Urea-Denatured MDH by Dilution and Dialysis.

Treatment[a]	Sp. Act.[b]	Reconstitution (percentage of control)
(1) Control	2750	100
(2) Urea, 8 M:		
(i) Dilution, 5×10^{-2}	0.8	<0.01
(ii) Dilution, 5×10^{-3}	8.0	≲0.3
(iii) Dilution, 5×10^{-4}	280	10
(3) Dialysis:		
(i) Control	700	100
(ii) From urea	750	106

[a] Treatment conditions: (1) MDH (0.3 mg) in 0.15 ml buffer A (see Materials) was added to 0.85 ml buffer A, incubated at 20° for 30 minutes, and assayed immediately. (2) MDH (0.3 mg) in 0.15 ml buffer A was added to 0.85 ml of 9.4 M urea, pH 8.2, and incubated at 20° for 30 min. (i) A sample of treatment 2 (0.05 ml) was assayed in a final volume of 1 ml. The final urea concentration was 0.4 M. (ii) A sample of treatment 2 was diluted 1:10 in 50 mM sodium phosphate buffer, pH 7.0, containing 0.1% BSA, at 4°, and a 0.05-ml diluted sample was assayed immediately. The final urea concentration was 0.04 M. (iii) Same as set 2ii except 1:100 dilution. The final urea concentration was 0.004 M. (3) Solutions of MDH from treatments 1 and 2 were dialyzed for 36 hr against 100 ml of buffer A at 4° in acetylated dialysis tubing and diluted as described in treatment 2iii prior to assay. (i) From treatment 1. (ii) From treatment 2. Final urea concentration <1.6 μM. [b] μmoles NADH oxidized min^{-1} (mg protein)$^{-1}$ at 25°.

TABLE IV: Reconstitution of Heat-Denatured MDH with Urea and Dialysis.

Treatment[a]	Sp. Act.[b]
(1) Control (unheated, undialyzed)	2500
(2) Heated, 100°, 10 min.	0
(3) Heated + 8 M urea + dialysis	2650
(4) Control (unheated, dialyzed)	2200

[a] Treatment conditions: (1) MDH (2.24 mg/ml) in buffer A at 4°. (See Materials for composition of buffer A.) (2) MDH (1.12 mg) in 0.5 ml buffer A was heated at 100° for 10 min in a stoppered tube and cooled in an ice bath. A flocculent, copious precipitate formed. (3) To the solution of treatment 2, 2.8 ml of 9.4 M urea, pH 8.2, was added. The flocculent precipitate dissolved immediately. The solution was incubated at 20° for 30 min and dialyzed against 200 ml of 50 mM sodium phosphate buffer, pH 7.0, for 36 hr at 4° in acetylated dialysis tubing. (4) The solution from treatment 1 was dialyzed as in treatment 3. [b] μmoles NADH oxidized min^{-1} (mg protein)$^{-1}$ at 25°.

Discussion

Neurospora malate dehydrogenase is a compact spherical molecule with a molecular weight of 54,000 (Munkres and Richards, 1965b). Comparative observations of amino acid replacement and sedimentation constants of mutant forms of MDH, together with theoretical genetical considerations, lead to the proposal that this enzyme is constructed from sequentially nonidentical polypeptides in the structure $\alpha\alpha\alpha\beta$ (Munkres and Richards, 1965a). Experiments reported here and in the accompanying paper lend additional support to the previous proposal.

The process of reversible dissociation of malate dehydrogenase from acid is viewed in terms of the reaction

$$\alpha\alpha\alpha\beta \underset{\text{pH 6.8}}{\overset{\text{pH 2.8}}{\rightleftharpoons}} \alpha\alpha\alpha + \beta$$

The native enzyme apparently has only one active site, as evidenced from ultraviolet spectrophotometric

titration with substrates in the formation of binary and ternary complexes (Munkres and Richards, 1965b). This conclusion has more recently been confirmed by spectrofluorimetric titrations of MDH with NAD (K. D. Munkres, unpublished). Thus the "catalytic monomer" [by the terminology suggested by Reithel (1963)] is the tetrameric form of the protein. Inactivation, accompanied by dissociation, of the protein by acid denaturation thus appears to indicate that a cooperative interaction and folding of the four polypeptide chains is essential for the formation of a single active site. It is yet uncertain whether the active site is formed by the folding of only one chain which is stabilized through interaction with the other three or whether residues from all four polypeptide chains participate directly in the catalytic function.

As expected for a process involving the two polypeptide components α_3 and β, the reconstitution of activity is influenced by the concentration of denatured protein. The extent of enzyme reconstitution at effectively infinite time as a function of the initial concentration of denatured protein (in the range 0.01–1 mg/ml) apparently reflects the equilibrium in the type reaction $A + B \rightarrow C$, where the reactants are present in equimolar concentration. At protein concentrations higher than 1 mg/ml the observed decrease in rate of reconstitution with increasing concentration may indicate the occurrence of secondary reactions involving illegitimate reassociations of the subunits. At very low concentrations (1–10 μg/ml) there is little reconstitution of activity. These results are similar to those of Levinthal *et al.* (1962) on the reconstitution of alkaline phosphatase and of Stellwagen and Schachman (1962) on the reconstitution of aldolase. The observations with these three multichain enzymes may be contrasted with those for the restoration of ribonuclease activity from the denatured, reduced single polypeptide chain. For this enzyme, extremely low concentrations favor the reconstitution process since intramolecular disulfide bonds form and there is a long lag period before activity is regenerated by shuffling of the disulfide bonds (Epstein *et al.*, 1962). These considerations indicate that the process of reconstitution of MDH, like that of alkaline phosphatase and aldolase, involves noncovalent interactions that are sufficient to direct and maintain the proper conformation. Additional and more direct evidence supporting this conclusion is indicated by observations of the effects of mercaptoethanol on reconstitution and by analysis of the free sulfhydryl groups of the protein.

Mercaptoethanol is essential for maximal enzymatic activity and for the complete reconstitution of activity after acid denaturation. Since all of the sulfhydryl groups are free in the active enzyme (Munkres and Richards, 1965a; Munkres, 1965), the absence of mercaptoethanol may allow the formation of illegitimate disulfide groups by oxidation prior to acid denaturation and during the denaturation–reconstitution process with concomitantly incorrect reassociation of the polypeptides.

It is now well established that a number of enzymes can recover activity after denaturation with high urea concentration. Although the exact nature of forces involved in denaturation of proteins by urea is still controversial, it has long been known that considerable disruption of the secondary and tertiary structures and a partial unfolding of the polypeptide chains obtains in urea denaturation (Kauzmann, 1959). Although the present experiments on the reconstitution of MDH activity after urea denaturation offer no clues as to the mechanism of the dissociation or reconstitution processes, in the accompanying paper results are presented that indicate that the α-trimer is completely and irreversibly dissociated to the monomer after denaturation in 8 M urea with strong mercaptoethanol, followed by treatment with iodoacetamide.

The renaturation by urea treatment of heat-denatured, inactive, insoluble enzyme was first observed by Perrin and Monod (1963) with β-galactosidase. These authors interpreted this phenomenon by the hypothesis that the "irreversibility" of a denatured state may not be due to the lesser thermal stability of the native state, but rather to a "freezing" of the denatured state by formation of illegitimate intermolecular bonds. According to this interpretation, renaturation of heat-denatured enzyme by urea followed by dialysis was interpreted as comparable to the renaturation of DNA by annealing at intermediate temperatures, whereas the fast removal of urea by dilution may not allow time for the proper bonds to be formed in the proper order, in analogy to the nonrenaturation of DNA after fast cooling.

The aim of the present experiments has not been to examine rigorously the secondary and tertiary structures formed in denaturation and reconstitution but rather to examine this subject from the point of view of whether some changes occurring with loss of enzymatic activity may lead to products which have physiological or genetical relevance. Numerous and recent investigations of the reversible denaturation and inactivation of enzymes support the hypothesis that the folding process by which a protein arrives at its three-dimensional configuration is essentially a thermodynamic one and that no genetic information, other than that present in the amino acid sequence of the protein, is required [for a recent review, see Reithel (1963)]. One corollary of the "thermodynamic" hypothesis, that certain amino acid replacements at vital points of the sequence for the determination of the higher structural orders may lead to marked configurational alterations of the protein molecule, was experimentally demonstrated in studies of mutant forms of *Neurospora* MDH (Munkres and Richards, 1965a). In the present experiments, the fact that the polypeptide subunits can interact even in the presence of foreign proteins (as noted by the reconstitution of enzyme activity from crude extracts) and at rates nearly identical with those observed with pure enzyme illustrates the precision of recognition that must be intrinsic to the amino acid sequence of the polypeptide chains. This observation is of physiological and genetical relevance in relation to the intercistronic complementation observed *in vivo* with the malate mutants (Munkres *et al.*, 1965). Mutant enzymes,

differently defective in either the α or β polypeptides and, consequently, differentially defective in conformation, may indeed form hybrid enzyme molecules during *in vivo* complementation that are functionally and conformationally more comparable to the native enzyme.

In pursuance of the relation between the *in vivo* and *in vitro* assembly of the polypeptide chains of MDH, the calculated rate of synthesis *in vivo* is 36 mμmoles $l.^{-1}$ min^{-1} at 20° (see Appendix). For this rate to obtain *in vitro*, the sum of the concentration of α_3 and β monomers would be 24 μg/ml, a concentration that is suboptimal for the *in vitro* system. Additional correlation of the *in vivo* and *in vitro* rates is not presently possible since nothing is known of the intracellular concentration or distribution of MDH subunits, the intracellular pH at the site(s) of assembly, or even whether the synthesis proceeds at maximal possible rates *in vivo*. However, it should be noted that *Neurospora* MDH may be an exceptionally favorable enzyme for studies of enzyme synthesis in subcellular systems *in vitro*, not only because of its relatively high turnover number and the availability of ultramicrofluorimetric enzyme assays but also because of the amenability of this enzyme to genetic analysis.

Appendix

The rate of formation of *Neurospora* MDH *in vivo* is calculated as follows. The total extractable protein from dry *Neurospora* mycelia is about 20% of the dry weight (K. D. Munkres, unpublished). The weight of intracellular water is 2.54 times the dry weight of mycelia (Slayman and Tatum, 1964). The concentration of MDH in crude extracts is 0.3% of the extractable protein, and the molecular weight is 54,000 (Munkres and Richards, 1965b). Assuming the density of intracellular water is unity and assuming MDH is uniformly distributed throughout the intracellular space, the intracellular concentration of MDH is

$$(\text{MDH}) = \frac{(0.003)(0.20)}{2.54(5.4 \times 10^4)} \times 10^3 = 4.37\ \mu\text{M}$$

Since the generation time of *Neurospora* is 2.3 ± 0.1 hr at 27°, the Q_{10} is 2.5 (Slayman and Tatum, 1964), and the specific activity of MDH remains essentially constant in extracts of exponentially growing mycelia (K. D. Munkres, unpublished), the steady-state rate of synthesis of MDH *in vivo* at 20° is

$$\frac{d(\text{MDH})}{dt} = \frac{2(4.37 \times 10^{-6}\ \text{moles}\ l.^{-1})}{242\ \text{min}}$$

$$= 36\ \text{m}\mu\text{moles}\ l.^{-1}\ \text{min.}^{-1}$$

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