The Hybridization of Glyceraldehyde 3-Phosphate Dehydrogenases from Rabbit Muscle and Yeast

KINETICS AND THERMODYNAMICS OF THE REACTION AND ISOLATION OF THE HYBRID

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A kinetic and thermodynamic study was made of the formation of the hybrid (R_2Y_2) glyceraldehyde 3-phosphate dehydrogenase from the yeast (Y_4) and rabbit (R_4) enzymes. The values of the thermodynamic parameters for the equilibrium between R_4 , Y_4 and R_2Y_2 suggest that the R_2-R_2 and Y_2-Y_2 interactions are similar. However, the failure to observe the RY_3 and R_3Y hybrids is interpreted in terms of differences at the interfaces of the $R-R$ and $Y-Y$ interactions (the glyceraldehyde 3-phosphate dehydrogenase molecule being regarded as a dimer of dimers). The kinetics of formation of the R_2Y_2 hybrid were studied and a model was proposed to account for the results. Best-fit values for the rate constants of the individual steps were evaluated by computer simulation, and the rate-limiting steps were identified as the dissociation of tetramers to dimers. It is proposed that the cleavage plane for dissociation of the tetramers corresponds to the region of low electron density through the centre of the molecule in the X-ray-crystallographic structure for human glyceraldehyde 3-phosphate dehydrogenase (Watson *et al.*, 1972), which is probably the plane containing the Q and R axes in the lobster enzyme (Buehner *et al.*, 1974). The R_2Y_2 hybrid was isolated in milligram amounts by ion-exchange chromatography and its rate of reversion to the native enzyme was shown to be consistent with the kinetic model proposed from the hybrid-formation experiments.

The present paper represents part of a continuing study of subunit interactions in glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) as approached through the study of kinetic and thermodynamic aspects of subunit interchange.

Hybridization studies have provided valuable information about quaternary structure and subunit interactions in oligomeric proteins. Most of these studies have involved hybridization of different forms of a protein from a single species, for example: lactate dehydrogenase (Markert, 1963; Jaenicke, 1970), creatine kinase (Dawson *et al.*, 1965), aldolase (Penhoet *et al.*, 1966, 1967) and haemoglobin (Antonini *et al.*, 1962; Huehns *et al.*, 1962*a,b*; Guidotti, 1967). Other studies have involved hybridization of a chemically modified protein with the native species, as with haemerythrin (Keresztes-Nagy *et al.*, 1965), aldolase (Meighen & Schachman, 1970*a*) and glyceraldehyde 3-phosphate dehydrogenase (Meighen & Schachman, 1970*b*). In the latter study three hybrid species were reported, as expected for a molecule comprising four identical polypeptide chains (Harrington & Karr, 1965). However, hybridization of yeast and rabbit glyceraldehyde 3-phosphate dehydrogenases (designated R_4 and Y_4 respectively) gave only a single observable hybrid species, designated R_2Y_2 , which was enzymically active and com-

prised two subunits of the rabbit enzyme and two of the yeast (Spotorno & Hollaway, 1970; Kirschner & Schuster, 1970). Spotorno & Hollaway (1970) concluded that the failure to observe three hybrid species, although both the yeast enzyme (Harris & Perham, 1965) and rabbit enzyme (Harrington & Karr, 1965) comprise four identical polypeptide chains, indicates that the native yeast and rabbit glyceraldehyde 3-phosphate molecules must be regarded as isologous rather than heterologous tetramers (Monod *et al.*, 1965). This conclusion is consistent with the molecular 222 symmetry of the enzymes from lobster muscle (Watson *et al.*, 1972).

Later work (K. Suzuki & J. I. Harris, personal communication) has shown that it is possible to obtain the RY_3 and R_3Y hybrids under different conditions, although they form to a much smaller extent than the R_2Y_2 hybrid, i.e. are less thermodynamically stable. Other reports of hybridization of glyceraldehyde 3-phosphate dehydrogenases have described the formation of three hybrid species between the rabbit and salmon enzymes (Leberherz *et al.*, 1973) and between the ascaris and rabbit enzymes (Kochman *et al.*, 1974).

The present paper is concerned with a kinetic and thermodynamic investigation of the formation of the R_2Y_2 hybrid species from native rabbit skeletal-

muscle and native yeast glyceraldehyde 3-phosphate dehydrogenases (designated R_4 and Y_4 respectively). The isolation of the R_2Y_2 hybrid is also reported as well as kinetic aspects of its rate of reversion to an equilibrium mixture of the R_4 , R_2Y_2 and Y_4 species. The results are discussed in terms of a model for the hybridization process.

Materials and Methods

Materials

Except where indicated analytical-grade reagents were obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). DL-Glyceraldehyde 3-phosphate diethyl acetal barium salt, NAD^+ and NADH (both grade 1) were purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). Cleland's reagent (dithiothreitol, A grade) was obtained from Calbiochem (Los Angeles, Calif., U.S.A.). Sephadex G-50 (coarse grade) and DEAE-Sephadex A-50 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Enzyme purifications

Yeast glyceraldehyde 3-phosphate dehydrogenase was prepared from commercial baker's yeast by using a slight modification of the method of Krebs (1952). All solutions contained 5 mM-EDTA and the filtration steps were replaced by centrifugation at 23000g for 30 min. The specific activity of preparations was between 140 and 170 units/mg measured by the standard assay method described by Trentham (1968); 1 unit represents 1 μ mol of NADH formed/min.

Glyceraldehyde 3-phosphate dehydrogenase was isolated from rabbit skeletal muscle by a modification of the method of Ferdinand (1964). The initial muscle extract was obtained by blending the muscle in the 50 mM-EDTA solution (see Ferdinand, 1964) at 4°C for 1 min in a Waring blender. Refractionation of the enzyme was by the method of Amelunxen & Carr (1967). The specific activity of preparations varied between 160 units/mg and 210 units/mg measured by the assay described by Trentham (1968). The E_{280}/E_{260} ratio of the enzyme measured in a 0.01 M-sodium phosphate buffer, pH 7.8, was 1.2 ± 0.02 and this increased to 1.3 ± 0.03 on desalting by passage through a column (10 cm $\times$ 1 cm) of Sephadex G-25 equilibrated with the phosphate buffer. The enzyme was found to contain less than 0.2 mol of reducible NAD^+ /mol of enzyme as determined from the E_{340} change in a solution containing enzyme, 3 mM-D-glyceraldehyde 3-phosphate and 10 mM-sodium arsenate in 0.05 M-tetrasodium pyrophosphate, adjusted to pH 8.5 with aq. HCl. It is probable that the E_{280}/E_{260} ratio is due to bound ADP-ribose, which is a result of

the isolation procedure (Bloch *et al.*, 1971). However, no bound acyl groups were present in the preparations as indicated by the observation that there were 4 molecules of NAD^+ reduced/molecule of enzyme in the presence of 7 mM- NAD^+ and 3 mM-D-glyceraldehyde 3-phosphate in 50 mM-imidazole, pH 7.0, containing 0.1 M-KCl, 5 mM-dithiothreitol and 1 mM-EDTA, in the absence of an external acceptor such as phosphate or arsenate. The enzyme, prepared as described above, exhibited the same properties in hybridization experiments as a preparation that had been precipitated by addition of $(NH_4)_2SO_4$ (solid) from a solution containing a 16-fold molar excess of NAD^+ and arsenate in 5 mM-EDTA adjusted to pH 7.6 with aq. NH_3 and then treated with 5 mg of activated charcoal/mg of enzyme to remove co-enzyme. This procedure gave an enzyme solution with an E_{280}/E_{260} ratio of 1.90.

Before use, portions of the crystalline enzyme suspended in 80% satd. $(NH_4)_2SO_4$ were centrifuged for 30 min at 38000 g. The pellets were dissolved in 0.01 M- NaH_2PO_4 , containing 1 mM-dithiothreitol, and 1 mM-EDTA, adjusted to pH 7.8 with NaOH (hereafter referred to as the pH 7.8 phosphate buffer) and $(NH_4)_2SO_4$ was removed by passage through a column (12 cm $\times$ 0.6 cm) of Sephadex G-50 equilibrated with the same buffer. Concentrations of the yeast and rabbit enzymes were measured spectrophotometrically, by using a value of $E_{1cm}^{1\%} = 9.2$ for the rabbit enzyme (Ferdinand, 1964) and $E_{1cm}^{1\%} = 10.0$ for the yeast enzyme with $E_{280}/E_{260} = 1.47$ (calculated from the extinction coefficients of Kirschner *et al.*, 1971).

Hybrid formation experiments

Solutions of the two enzymes were mixed and diluted to the required concentration with the pH 7.8 phosphate buffer. After incubation of the mixture for the given times, starch-gel electrophoresis was carried out for 3 h at 25 V/cm in 0.1 M-Tris base-0.063 M-boric acid-2 mM-EDTA buffer, pH 8.7 (Rosemeyer & Huehns, 1967). Gels were sliced into two and stained for protein with 1% Amido Black 12B in 7% (v/v) acetic acid and destained in acetic acid-methanol-water (1:5:5, by vol.). Staining for enzyme activity was carried out as described by Spotorno & Hollaway (1970).

Photographs of the protein-stained gels were taken and the negatives were scanned with a Mk III B Joyce-Loebl microdensitometer. The areas under the peaks of the densitometer traces were evaluated either by triangulation or by planimetry, and the amount of protein in a given band was determined by using a calibration curve constructed from traces of photographs of gels containing measured amounts of enzyme. No significant difference in the distance of

migration of yeast or rabbit glyceraldehyde 3-phosphate dehydrogenase was observed in the concentration range from 1–25 mg/ml.

Hybrid isolation

A solution (2 ml) of yeast and rabbit glyceraldehyde 3-phosphate dehydrogenase, each at 10 mg/ml in the pH 7.8 phosphate buffer, was dialysed against 5 litres of the same buffer for 14 h at 4°C, and then chromatographed on a column (18 cm × 1.1 cm) of DEAE-Sephadex A-50 equilibrated with the dialysis buffer. The column was developed with the equilibration buffer until the beginning of the elution of the first peak, at which point a linear gradient of NaCl was applied such that the NaCl concentration changed at a rate of 3 mM/ml of effluent. The flow rate was 17 ml/h and fractions of volume 2 ml were collected. Peak fractions were concentrated by ultrafiltration through a Diaflo membrane [Amicon, N.V., Oosterhout (NB), The Netherlands] at 2.06×10^4 Pa. All operations were at 4°C unless specified otherwise.

Results

Kinetic and thermodynamic aspects of hybrid formation

Fig. 1 shows electrophoretograms of mixtures of yeast (designated Y_4) and rabbit glyceraldehyde 3-phosphate dehydrogenase (designated R_4) each at 6 mg/ml, preincubated at different temperatures for different times. Only a single hybrid band can be seen, which is identified as species R_2Y_2 from its relative distance of migration. This confirms earlier findings of Spotorno & Hollaway (1970) and Kirschner & Schuster (1970). The fact that the protein-staining bands are well defined suggests that there is little inter-conversion of the three species R_4 , Y_4 and R_2Y_2 during the time of electrophoretic separation and this is confirmed by the failure to observe any significant hybrid formation in solutions of the R_4 and Y_4 species mixed immediately before electrophoresis (Fig. 1c). All the protein-staining bands showed enzymic activity when the stain procedure described in the Materials and Methods section was used.

From the areas under the peaks in the densitometric traces in Figs. 1(a) and 1(b) percentage compositions were calculated for the ratio $R_4:R_2Y_2:Y_4$ of 29:39:32 for an incubation for 3 h at 37°C and 40:22:38 for a 21 h incubation at 0°C. The greater extent of conversion into hybrid after incubation for a shorter period at the higher temperature could be an expression of the kinetic or thermodynamic properties of the hybridization process, so an investigation was made of the rate of attainment and position of the equilibrium between species R_4 , and Y_4 and R_2Y_2 at different temperatures.

Fig. 2 shows the time-courses of formation of the R_2Y_2 and Y_4 species in mixtures of the yeast and rabbit enzymes incubated in the pH 7.8 phosphate buffer at 28°C. Data are included from two experiments, one with the concentration of each enzyme at 6 mg/ml (41 μ M, assuming a molecular weight of 145000) and the other at 1.2 mg/ml (8.3 μ M). Within experimental error the time-courses at the two concentrations are identical. The simplest kinetic scheme that can be proposed to account for the hybridization process is given by Scheme 1. The dissociation constant for the tetramer–dimer interconversion for the rabbit enzyme is about 0.2 μ M (Hoagland & Teller, 1969) and that for the yeast enzyme is probably not greatly different from this (Kirschner & Schuster, 1970), so that with enzyme solutions of both 41 and 8.3 μ M there will be relatively low concentrations of dimers compared with tetramers (less than 16% even with 8.3 μ M-enzyme). This suggests that during the hybridization process it is reasonable to assume that the rates of change of dimer concentrations with time are small compared with the rates of change of tetramer concentrations, i.e. a steady-state treatment is applicable. The further assumption that $k_{+r} = k_{+y}$ and $k_{-r} = k_{-y}$ gives a system which can be solved analytically to give an expression for the concentration of hybrid formed ($[R_2Y_2]_t$) at any given time t in an equimolar mixture of yeast and rabbit enzymes at initial concentrations $[R]_0$. The result is given by eqn. (1):

$$[R_2Y_2]_t = A[1 - \exp(-k_1^{\text{obs}} t)] \quad (1)$$

where:

$$A = \frac{2k_{-r} \cdot k_{+h} \cdot [R]_0}{(2k_{+r} \cdot k_{-h} + k_{+h} \cdot k_{-r})} \quad (2)$$

and:

$$k_1^{\text{obs}} = k_{-h} + \frac{k_{+h} \cdot k_{-r}}{2k_{+r}} \cdot \frac{1}{1 + \frac{k_{+h}}{2k_{+r}}} \quad (3)$$

Thus, given the assumptions, the hybridization process will be a first-order approach to equilibrium with rate constant k_1^{obs} and a concentration A of hybrid at equilibrium. It is noteworthy that in the event that $k_{-r} = k_{-h}$ and $k_{+h} = 2k_{+r}$ (the collision frequency between different molecular species is twice that between similar species, given components at the same concentrations), then the expression for k_1^{obs} in eqn. (3) simplifies to k_{-r} and the equilibrium concentration of hybrid (A) becomes $[R]_0$, i.e. the equilibrium concentrations of species R_4 , R_2Y_2 and Y_4 will be $[R]_0/2$, $[R]_0$ and $[R]_0/2$ respectively, and the rate of the hybridization is governed by the dissociation-rate constant for the tetramer-into-dimer conversion.

The experimental data of Fig. 2 are not sufficiently precise to be able to establish unequivocally that the

Fig. 1. *Starch-gel electrophoretograms and densitometric traces of mixtures of rabbit and yeast glyceraldehyde 3-phosphate dehydrogenases*

Both enzymes were at 6 mg/ml in each experiment and the preincubations were carried out in pH 7.8 phosphate buffer. (a) Incubation for 3 h at 37°C; (b) for 21 h at 0°C; (c) electrophoresis immediately after mixing. Electrophoresis and densitometric analysis were carried out as described in the text.

reaction is a first-order approach to equilibrium. However, a first-order plot of the data was made from which a value of $k_1^{\text{obs.}} = 9.5 \pm 3.0 \times 10^{-5} \text{ s}^{-1}$ was calculated.

To obtain eqn. (1) it was necessary to assume identical values for the dissociation-rate constants for the rabbit and yeast tetramers to dimers (k_{-r} and k_{-y} respectively). To test this assumption two separate

incubation experiments were conducted, one in which the concentration of the rabbit enzyme was five times that of the yeast enzyme and another in which these concentrations were reversed. The electrophoreto-

Fig. 2. Observed and computed time-courses for breakdown of species Y_4 and formation of species R_2Y_2 in equimolar mixtures of R_4 and Y_4 .

The native enzymes were incubated together at pH 7.8 and 28°C for given times and the mixtures subjected to electrophoretic analysis to determine the relative amounts of species Y_4 and R_2Y_2 . □ and ■ denote the percentages of species Y_4 and R_2Y_2 respectively in an incubation mixture initially containing 8.3 μM - R_4 and $-Y_4$. ○ and ● represent the percentages of species Y_4 and R_2Y_2 respectively at different times when the initial concentrations of R_4 and Y_4 were 41 μM (percentages refer to the respective initial concentrations, which were calculated on the basis of molecular weights of 145000). The least-squares best-fit computed time-courses (by using program CHEKMAT) are given for Y_4 and R_2Y_2 . The best-fit values of the rate constants are given in Scheme 1. The s.d. of the points (least squares) is 5.7%.

grams of the mixtures incubated for 3 h at 28°C in the pH 7.8 phosphate buffer are shown in Fig. 3. Densitometric analysis of these electrophoretograms showed that three times as much hybrid had formed in the experiment with a fivefold excess of species R_4 as in that with a fivefold excess of species Y_4 .

The simplest interpretation of this result is that the value of k_{-y} in Scheme 1 is about three times that of k_{-r} , in which case eqn. (2) is not strictly applicable to the hybridization system since it requires that $k_{-r} = k_{-y}$. To investigate further the kinetic behaviour of Scheme 1 a digital computer simulation was set up by using the programs CHEK and CHEKMAT devised by Curtis & Chance (1972). By using program CHEKMAT the values of rate constants in Scheme 1 giving least-squares best fit of the data of Fig. 2 were determined. These values are included in Scheme 1. Fig. 2 shows a comparison of the observed and theoretical progress curves, from which it can be seen that

Fig. 3. Effect of varying the relative concentrations of species R_4 and Y_4 on the amount of hybrid formed in a given time

The two photographs show electrophoretograms of 3 h incubation mixtures (a) with initial $[R_4] = 11 \mu\text{M}$ and $[Y_4] = 55 \mu\text{M}$, (b) with $[R_4] = 55 \mu\text{M}$ and $[Y_4] = 11 \mu\text{M}$. Incubations were in the pH 7.8 phosphate buffer at 28°C and electrophoresis and protein staining were carried out as described in the Materials and Methods section.

Scheme 1. Kinetic model for dimer exchange between rabbit (R_4) and yeast (Y_4) glyceraldehyde 3-phosphate dehydrogenases

The values of the rate constants given in parentheses were determined as described in the text. Y_2 and R_2 represent dimeric species and R_2Y_2 the hybrid tetrameric species.

the data can be fitted to Scheme 1 within the considerable experimental error. Merely finding an approximate fit of the data to the Scheme does not of course prove that the Scheme or the values of the rate constants are correct. However, the most significant feature of the time-courses shown in Fig. 2 is that they are closely similar at different initial enzyme concentrations. This similarity is consistent with Scheme 1.

It was established that it was not possible to obtain a good fit to the data with widely different values of the rate constants for the tetramer-into-dimer conversions. In particular, making the values of these first-order rate constants larger than those given in Scheme 1 by a factor of 10^3 gave a theoretical progress curve for hybrid formation with a half-life of about 16s, a result which is clearly inconsistent with observations (see Fig. 1c). Further simulation studies revealed that it is the values of the dissociation rate constants k_{-r} , k_{-y} and k_{-h} that govern the rate of hybrid formation, the rate being relatively insensitive to the absolute values of the second-order rate constants although their ratios affect the predicted distribution of species concentrations at equilibrium. In this case we have been unable to calculate values either for the second-order-recombination constants (k_{+r} , k_{+y} and k_{+h}) or, consequently, dissociation constants for the tetramer-dimer conversions.

Isolation and stability of the R_2Y_2 hybrid

It seemed likely that the properties of the isolated R_2Y_2 hybrid would reveal further information about the nature of the hybridization process and provide a further test of the validity of Scheme 1. The method for isolation of the hybrid is described in the Materials and Methods section, and Fig. 4 shows the elution profile of the chromatographic separation of the components of the hybridization mixture. This Figure also depicts the densitometric traces from an electrophoretogram of the concentrated peak fractions, demonstrating that the procedure effectively separates the R_2Y_2 hybrid from the native enzymes.

The availability of the isolated R_2Y_2 hybrid made it possible to follow the time-course of its reversion to an equilibrium mixture of R_4 , R_2Y_2 and Y_4 . This time-course is shown in Fig. 5, which also depicts the least-squares best-fit of the data obtained by using the program CHEKMAT with Scheme 1 as the kinetic model. The calculated values of the three dissociation rate constants of $k_{-r} = 1.9 \times 10^{-5} \text{ s}^{-1}$, $k_{-y} = 5.7 \times 10^{-5} \text{ s}^{-1}$ and $k_{-h} = 4.8 \times 10^{-5} \text{ s}^{-1}$ determined from the reversion experiment, although one-third to one-quarter of those obtained by following the reaction in the opposite direction, are not so widely different to invalidate the model, especially considering the low

Fig. 4. Chromatographic resolution of a hybridized mixture of species R_4 , R_2Y_2 and Y_4

A mixture (2ml) containing the yeast and rabbit enzymes each at 10mg/ml was incubated in the pH7.8 phosphate buffer at 4°C for 14h and then applied to a column (18cm $\times$ 1.1cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. Fractions (2ml) were collected and the elution profile (solid line) recorded continuously by monitoring % transmission at 254nm. After elution of the first peak with the equilibration buffer, a linear concentration gradient of NaCl was applied (broken line). Each peak fraction was concentrated separately as described in the Materials and Methods section, and electrophoretic analysis carried out as described in Fig. 1. The densitometric traces above each peak correspond to the electrophoretic analysis of the respective concentrates. These traces are in register with respect to the distance of migration from the origin and include an analysis of the hybridized mixture before chromatography. The left-hand trace is for the electrophoretogram of the mixture applied to the column. Peaks are designated I, II and III, counting from the left of the elution profile.

Fig. 5. Observed and computed time-courses for the breakdown of the R_2Y_2 hybrid to an equilibrium mixture of species R_4 , R_2Y_2 and Y_4

The isolated hybrid from peak II (Fig. 4) at 6mg/ml was incubated for different times in the pH 7.8 phosphate buffer at 28°C. The amount of hybrid remaining in the mixture was determined by electrophoretic and densitometric analysis as described in the Materials and Methods section. The solid line is the computed best fit to the data, obtained by using program CHEKMAT.

precision inherent in the method for following the reactions. A continuous method for measuring the hybridization progress would offer greater precision, but attempts to follow the reaction by using changes in u.v. absorption, fluorescence or pH value were unsuccessful.

Effect of temperature on the composition of equilibrium mixtures R_4 , R_2Y_2 and Y_4

Mixtures of rabbit and yeast enzymes were left for sufficient time to reach equilibrium with the R_2Y_2 hybrid and then the mixtures were subjected to electrophoretic analysis (see the Materials and Methods section). The equilibrium constant $K_{app.}$ at each temperature was calculated from the relationship: $K_{app.} = [R_2Y_2]^2/[R_4][Y_4]$ and the standard enthalpy change (ΔH_0) for the reaction was calculated by using the van't Hoff relationship:

$$\left(\log \left[\frac{(K_{app.})_{T_1}}{(K_{app.})_{T_2}} \right] = \frac{\Delta H_0 (T_2 - T_1)}{2.303 R \cdot T_1 T_2} \right)$$

The results are given in Table 1 from which a value of $\Delta H_0 = +25$ kJ/mol was calculated for the formation of hybrid. This unfavourable enthalpy change is compensated by a positive entropy change ($\Delta S_0' = 84$ J/°C per mol). The observation that the composition of equilibrium mixtures was independent of the

Table 1. Thermodynamic parameters for the equilibrium between yeast, rabbit and hybrid (R_2Y_2) dehydrogenases

Mixtures of yeast and rabbit glyceraldehyde 3-phosphate dehydrogenases, each at 6mg/ml in the pH 7.8 phosphate buffer, were incubated at different temperatures between 310° and 273°K until equilibrium was reached (the time taken varied from 3 to 48 h). The relative equilibrium concentrations of the three species R_4 , R_2Y_2 and Y_4 were then determined by electrophoretic analysis as described in the Materials and Methods section and the equilibrium constants were calculated from the relationship: $K_{app.} = [R_2Y_2]^2/[R_4][Y_4]$. A van't Hoff plot of this data gave a value of $\Delta H_0' = 25$ kJ/mol for hybrid formation. Hence a value of $\Delta S_0' = 84$ kJ/°C per mol was calculated.

	Temperature (°K)			
	273	284	301	310
$K_{app.}$	0.46	0.72	1.16	1.79
$\Delta G_0'$ (kJ/mol)	1.59	0.79	-0.71	-1.46

pH value in the range 6.5–8.0 (P_i buffers, $I = 0.03$ M) established that the temperature-dependence of $K_{app.}$ was not an indirect result of the change in pH value of the medium with temperature.

Effect of temperature on the rate of attainment of the hybridization equilibrium

The amount of R_2Y_2 hybrid formed in a fixed time at different temperatures was measured. Assuming that the hybridization process was a first-order approach to equilibrium, with the equilibrium positions at the different temperatures derived from the results of the preceding section, apparent first-order rate constants were calculated from single determinations. The values obtained through this procedure are given in Fig. 6 in the form of an Arrhenius plot from which an Arrhenius activation energy of $E_a = 84$ kJ/mol was calculated.

Hybridization under conditions known to lead to monomer formation

Hybridization was carried out under the conditions which lead to extensive monomer formation in both the rabbit enzyme (Constantinides & Deal, 1969) and the yeast enzyme (Stancel & Deal, 1969). A mixture of the enzymes each at 0.07 mg/ml in 0.1 M-Tris-HCl buffer, pH 8.5, containing 5 mM-ATP and 0.1 M-2-mercaptoethanol was incubated at 4°C for 15 h. Reassembly was effected by diluting 1:1 (v/v) with the same buffer containing 10% (w/v) sucrose and incubating at 25°C for 3 h. The solution was then concentrated to a total protein concentration of 3.2 mg/ml by ultrafiltration at 25°C (see the Materials and Methods section). Starch-gel electrophoresis of the

Fig. 6. Arrhenius plot of the temperature dependence of the apparent first-order rate constant for the formation of the R_2Y_2 hybrid from species R_4 and Y_4 .

Mixtures containing the yeast and rabbit enzymes, each at 6 mg/ml, were incubated for 10 min in the pH 7.8 phosphate buffer and the concentration of hybrid formed ($[R_2Y_2]_t$) was determined by electrophoretic analysis as described in the Materials and Methods section. Given the equilibrium constants at the different temperatures (Table 1) the equilibrium concentrations of the hybrid $[R_2Y_2]_{\infty}$ were calculated, and then the first-order rate constants were calculated from the relationship:

$$k_1^{\text{app}} = \frac{1}{t} \ln \left\{ \frac{[R_2Y_2]_{\infty}}{([R_2Y_2]_{\infty} - [R_2Y_2]_t)} \right\}$$

resulting concentrate revealed only three enzymically active bands, which were identified as species R_4 , R_2Y_2 and Y_4 from their relative distances of migration.

Discussion

It is well established that hybridization of the homotetrameric lactate dehydrogenases from skeletal muscle (M_4) and heart muscle (H_4) yields three hybrid species: MH_3 , M_2H_2 and M_3H in amounts that are not far removed from a binomial distribution (for example, see Jaenicke, 1970). Since the rabbit and yeast glyceraldehyde 3-phosphate molecules also comprise four identical polypeptide chains (Perham, 1969; Harris, 1970; Jones & Harris, 1972) it was unexpected that only a single hybrid (R_2Y_2) could be observed on starch-gel electrophoresis in the Tris-borate-EDTA buffer system, even after long periods of hybridization under conditions where both enzymes are known to give monomeric species. Spotorno & Hollaway (1970) and Kirschner & Schuster (1970) also observed only a single hybrid species on starch-gel electrophoretograms after hybridization of the yeast and rabbit enzymes under similar conditions to those used in the present study.

The failure to observe the formation of the R_3Y and RY_3 hybrids may be attributed to the use of starch-gel electrophoresis in Tris-borate-EDTA buffers (see the Materials and Methods section) since recent work (K. Suzuki & J. I. Harris, personal communication) has shown that it is possible to detect protein-staining bands corresponding to the R_3Y , R_2Y_2 and RY_3 species on cellulose acetate strip electrophoretograms of mixtures of the rabbit and yeast enzymes which had been hybridized, either by freezing and thawing, or by incubation in the presence of 3 M-NaCl. Nevertheless only small amounts of the R_3Y and RY_3 species were detectable. We have confirmed this observation (H. H. Osborne & M. R. Hollaway, unpublished work) and find that incubation of a mixture of the rabbit and yeast enzymes (each at 3 mg/ml) for 6 h at 28°C in the pH 7.8 phosphate buffer (see the Materials and Methods section) gives a ratio of amounts of the species R_4 : R_3Y : R_2Y_2 : RY_3 : Y_4 of about 1:<0.1:1:<0.1:1 (as determined densitometrically from cellulose acetate strip electrophoretograms run for 3 h in 50 mM- KH_2PO_4 adjusted to pH 7.0 with KOH). Thus although the R_3Y and RY_3 species form, they do so in small amounts compared with the R_2Y_2 species.

The relative instability of the R_3Y and RY_3 species can be explained in at least two ways both of which depend on the structure of the glyceraldehyde 3-phosphate molecule being regarded as a 'dimer-of-dimers' (see below). The discussion is based on Scheme 2 which is a model for the formation of all molecular species in the incubation mixture. The first possibility is that there are significant differences at the interfaces of intermonomer contact within the R_2 and Y_2 dimers. These differences could render the RY dimer unstable with respect to the R_2 and Y_2 dimers and in turn confer instability on the R_3Y and RY_3 species, both of which contain RY dimers. Similarly the $(RY)_2$ tetramer, which is essentially different from the R_2Y_2 tetramer, would also be relatively unstable with respect to any species not containing the R - Y intradimer interaction. A second possibility is that the R_2 - RY and Y_2 - RY dimer-dimer interactions required to make the R_3Y and RY_3 species are weaker than the R_2 - R_2 and Y_2 - Y_2 interactions which determine the tetrameric structure of the native enzyme molecules. Superficially this possibility seems less likely since the free energy of interaction between R_2 and Y_2 in the R_2Y_2 dimer is similar to that between R_2 dimers in R_4 , and Y_2 dimers in Y_4 (see Table 1). However, an unfavourable R - Y interaction could lead to a modified structure for an RY dimer, which could in turn decrease the free energy of interaction of this species with R_2 or Y_2 . This case represents a combination of the two possibilities presented above, although the instability of the R_3Y and RY_3 species arises essentially from the RY instability.

The postulated difference between the intradimer subunit interactions in the yeast and rabbit enzymes could be the cause of the large differences in their affinity for NAD^+ as well as the shapes of the respective saturation curves. Thus the rabbit enzyme binds NAD^+ with dissociation constants ranging from less than 10pM for the first site to about $26\mu\text{M}$ for the fourth (Conway & Koshland, 1968) whereas, whichever set of binding data is accepted, the yeast enzyme has a much lower affinity for NAD^+ with dissociation constants for each of the sites between $6\mu\text{M}$ and $4000\mu\text{M}$ (Cook & Koshland, 1970; Kirschner, 1971).

It is important in the context of the present study to explain why it is possible to detect small amounts of the R_3Y and RY_3 hybrids on cellulose acetate strip electrophoresis carried out in a phosphate buffer at pH7, but not at pH8.7 in the Tris-borate-EDTA buffer. Scheme 2 is a minimal kinetic scheme to describe the hybridization process involving all species and the loss of the minor bands on electrophoresis in the Tris-borate-EDTA buffer will be discussed in terms of this model. During electrophoresis any process which equilibrates rapidly on the time-scale of the separation will result in a perturbation in the concentrations of the species involved in that process. Thus relatively high rates of breakdown of species R_3Y to R_2 and RY and of species RY_3 to RY and Y_2 at pH8.7 would result in a 'melting out' of the R_3Y and RY_3 bands, whereas

the R_2Y_2 hybrid would be stable if there was a lower value of the rate constant for its decomposition to dimers. Slower rates of breakdown of the R_3Y and RY_3 hybrids in the pH7.8 phosphate buffer would account for the ability to observe these components after electrophoresis in this buffer. This is in accord with the evidence for an increasing tendency for rabbit muscle glyceraldehyde 3-phosphate dehydrogenase to dissociate at increasing pH values in the range pH8 to 10 (Hoagland & Teller, 1969; Smith & Schachman, 1973), and this could result from an increase in the value of the dissociation rate constant as the pH value is raised.

The foregoing interpretation is essentially similar to that proposed by Guidotti *et al.* (1963) to account for hybridization patterns of haemoglobins A and S. These authors pointed out that only two components would be observed on separation of a mixture of three species in equilibrium if the rate of attainment of the equilibrium was faster than the particular controlling rate of the separation process. The discussion developed in the next paragraph is also based on this concept.

We have also tested alternative kinetic models for consistency with the data presented in this study. One possibility, based on Scheme 2, is that the single hybrid band observed on starch-gel electrophoretograms has the structure $(\text{RY})_2$ rather than R_2Y_2 . If all dimer-tetramer exchange processes were rapid and all monomer-dimer exchanges slow on the time-

Scheme 2. Interconversion of all possible species formed by subunit exchange between rabbit and yeast enzyme tetramers (R_4 and Y_4 respectively)

Note that $(\text{RY})_2$ is essentially different from R_2Y_2 owing to the molecule being regarded as a dimer-of-dimers.

scale of electrophoretic migration then species R_2Y_2 , RY_3 (i.e. $RY:Y_2$) and R_3Y (i.e. $RY:R_2$) would not be observed since they would 'melt out' as a result of the perturbation of the rapidly relaxing equilibria. In this case the half-life for the formation of species $(RY)_2$ should decrease as the initial concentrations of the native enzymes are lowered, since this would (a) increase the percentage of the enzymes present as R_2 and Y_2 dimers, (b) thus increase the rate of monomer formation and (c) therefore increase the rate of species RY , and so $(RY)_2$, formation. Computer simulation of Scheme 2 by using the program CHEK (M. R. Hollaway, unpublished work) confirmed this conclusion. Since the time-courses are independent of the initial concentrations of the R_4 and Y_4 species (see Fig. 2) then, given the above assumptions, the possibility can be excluded that the central hybrid band is species $(RY)_2$. Therefore it must be species R_2Y_2 . The observation of a five-banded hybridization pattern (see above) adds further confirmation to this conclusion.

Another possible model for hybrid formation is one proposed by Kochman *et al.* (1974) to account for the hybridization patterns from mixtures of mammalian muscle (X_4) and *Ascaris suum* muscle (As_4) glyceraldehyde 3-phosphate dehydrogenases (Scheme 3). This model, which does not involve monomer formation, would give the RY dimer by a dissociation of the R_2Y_2 hybrid across a different cleavage plane from that of the first dissociation in the native molecules. Inspection of Scheme 3 shows that the half-life of formation of the R_3Y and RY_3 species should be independent of the initial concentrations of fixed ratios of the two enzymes. This is so because, although dilution increases the concentration of all dimeric species, it will not alter the ratio of the rates at which they reform the different tetrameric species. Computer simulation of Scheme 3 by using program CHEK confirmed this concentration independence (M. R. Hollaway & E. M. Chance, unpublished work). However, Kochman *et al.* (1974) found that the extent of formation of the As_3X and AsX_3 hybrids at a

given time increased at lower initial concentrations of As_4 and X_4 . This observation is inconsistent with Scheme 3. Therefore we conclude that, in the present work, the central hybrid band on starch-gel electrophoretograms corresponds to the R_2Y_2 dimer and that it is formed by a process represented by Scheme 1. Of course this Scheme is at best an oversimplification of what must be a highly complex process. However, it does suffice to encompass the observations made both from the investigation of the rate of formation of R_2Y_2 from a mixture of R_4 and Y_4 as well as the rate of reversion of R_2Y_2 to the native species.

The first-order rate constants of the order of $10^{-4}s^{-1}$, which limit the overall rate of the R_2Y_2 hybrid formation, are attributed to the formation of dimers from tetramers. In particular the dissociation of the rabbit tetramer, R_4 , would appear to be the most important rate-limiting process since in the experiment with an excess of R_4 over Y_4 a larger amount of hybrid was produced in a given time with the concentration of Y_4 greater than that of R_4 (see Fig. 3). However, it could be argued that the rate constant of about $10^{-4}s^{-1}$ is attributable to a slow isomerization (or an unfavourable equilibrium position) of dimers after a rapid dissociation, i.e. for the rabbit enzyme:

the R_2^* species representing the conformation of the dimer that is required before it can combine with a similarly derived Y_2^* species to form the $R_2^*Y_2^*$ hybrid. (This model could be checked by measuring the rate of combination of isolated R_2 dimers.) However, the concentrations of R_4 , R_2Y_2 and Y_4 in the hybridized equilibrium mixture are not far removed from the expected binomial distribution for the case where the free energy of interaction of R_2 with Y_2 in the R_2Y_2 dimer is the same as the R_2-R_2 and Y_2-Y_2 interaction energies in the native enzyme, suggesting that the conformation of the R_2 and Y_2 dimers are not significantly different in the hybrid and the native

Scheme 3. Kinetic model for the formation of the hybrid species R_3Y and RY_3

Formation of species R_2Y_2 proceeds as in Scheme 1 and R_2Y_2 then dissociates by cleavage along a different plane to give RY dimers which combine with R_2 or Y_2 dimers to give tetramers R_3Y and RY_3 .

enzymes. Thus the rate-limiting step in hybrid formation is considered to be the slow dissociation of tetrameric species. In this case the rate and extent of formation and breakdown of the R_2Y_2 hybrid, allows an investigation to be made of interactions in different liganded states of the enzymes (H. H. Osborne, & M. R. Hollaway, unpublished work). The R_2Y_2 hybrid molecule can thus be regarded as a complex between dimers derived from the yeast and rabbit enzyme molecules by cleavage of the tetramers along the interface of secondary, isologous interaction. This interface is identified with the region of low electron density between the two halves of the human glyceraldehyde 3-phosphate molecule described by Watson *et al.* (1972) and probably corresponds to the plane containing the Q and R axes designated by Buehner *et al.* (1974).

Although no direct measurement of the rate constants for dimer combinations could be made in the present study, a value for the dissociation equilibrium constant, K , for the tetramer-dimer conversion would enable an indirect estimate to be made from the relationship $K = k_{-r}/k_{+r}$, given the measured values of k_{-r} and k_{+r} (see Scheme 1). Hoagland & Teller (1969) reported values for this dissociation constant for the rabbit muscle enzyme of about $0.35 \mu M$ in a phosphate buffer at pH 7 and $5^\circ C$ and $1.7 \mu M$ in a pyrophosphate buffer at pH 8.5 at the same temperature. From the results given in Fig. 6 it can be calculated that the value of k_{-r} in the phosphate buffer pH 7.8 at $5^\circ C$, is about $1.5 \times 10^{-5} s^{-1}$, so that the value of k_{+r} is approx. $15 M^{-1} \cdot s^{-1}$. This value is surprisingly low, being four or more orders of magnitude smaller than the values for other macromolecular combination processes, e.g. the second-order rate constant for the combination of the pancreatic trypsin inhibitor with trypsin is about $1.1 \times 10^6 M^{-1} \cdot s^{-1}$ (Lazdunski & Vincent, 1972) and that for the association of the α and β chains of haemoglobin is about $2.5 \times 10^5 M^{-1} \cdot s^{-1}$ (Antonini & Brunori, 1971). The calculated value for k_{+r} is also clearly far less than the value of the theoretical diffusion-limited rate constant, which is about $2 \times 10^9 M^{-1} \cdot s^{-1}$ by substituting in the equation of Smoluchowski (1916, 1917) values of $10^{-6} cm^2 \cdot s^{-1}$ for the diffusion coefficient for the enzyme dimer (see Edsall, 1953) and 6 nm for the collision diameter (Watson & Banaszak, 1964; Watson *et al.*, 1972). If it is assumed that tetramer formation only occurs in collisions where (a) the centroid of one binding surface is within one-tenth of a binding-surface diameter of the other, (b) the orientation of the collision pair is within 10° of the dimer-dimer orientation in the native molecule then it can be calculated that only 1 in about 3×10^4 collisions will be productive. Conditions (a) and (b) probably represent overestimates of the steric requirements so that the latter value can be regarded as maximum.

Thus a 'reasonable' theoretical value for the rate constant k_{+r} is of the order of $10^5 M^{-1} \cdot s^{-1}$, which is about four orders of magnitude greater than the value calculated on the basis of the present work. This difference could be accounted for: (a) if the value of the rate constant for hybrid formation is not a measure of the rate constant for tetramer-into-dimer conversion, (b) if the value of the dissociation equilibrium constant for the rabbit enzyme tetramer-into-dimer conversion is several orders of magnitude smaller than the value of about $1 \mu M$ assumed in the calculation, (c) if combination of dimers (R_2^*) to tetramers (R_4) only proceeds via a conformation of the dimer (R_2) which is present in small amounts, e.g. eqn. (4):

It is noteworthy that Antonini & Brunori (1971) have reported a similar scheme for the combination of the α and β chains of haemoglobin where a bimolecular association of the chains is followed by a unimolecular rearrangement. Although the earlier discussion shows possibility (a) to be unlikely, it is not possible at present to discriminate between these possibilities.

The positive enthalpy change for the formation of the R_2Y_2 hybrid from the native enzymes ($\Delta H'_0 = 25 kJ/mol$) allied to the favourable entropy change ($\Delta S' = 84 kJ/^\circ C$ per mol) suggests that there is a slightly greater extent of hydrophobic bonding in the R_2-Y_2 interaction than in the interdimer interactions in the native enzymes (for example, see Kauzmann, 1959). However, the above results indicate that the differences are minor so that the interactions between the R_2 and Y_2 dimers in the hybrid closely resemble the R_2-R_2 and Y_2-Y_2 interactions.

It is noteworthy that there is a 68% identity in the amino acid sequences of yeast and pig muscle glyceraldehyde 3-phosphate dehydrogenases (Jones & Harris, 1972). Since there are not more than five differences in the sequences of the pig and rabbit enzymes (R. N. Perham & J. I. Harris, personal communication), there must also be about 68% identity in the sequences of the yeast and rabbit enzymes, i.e. there are about 32% differences. It would be surprising in the light of the present results if many of these differences were to be at the dimer-dimer interface, although significant differences would be predicted for residues at the monomer-monomer interface in the first isologous interaction which makes up the glyceraldehyde 3-phosphate dehydrogenase molecule.

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References

- Amelunxen, R. E. & Carr, D. O. (1967) *Biochim. Biophys. Acta* **132**, 256–259
- Antonini, E. & Brunori, M., (1971) *Haemoglobin and Myoglobin in the Reactions with Ligands* p. 121, North-Holland Publishing Co., Amsterdam
- Antonini, E., Wyman, J., Bucci, E., Fronticelli, C. & Rossi-Fanelli, A. (1962) *J. Mol. Biol.* **4**, 368–375
- Bloch, W., MacQuarrie, R. A. & Bernhard, S. A. (1971) *J. Biol. Chem.* **246**, 780–790
- Buehner, M., Ford, G. C., Moras, D., Olsen, K. W. & Rossmann, M. G. (1974) *J. Mol. Biol.* **82**, 563–585
- Constantinides, S. M. & Deal, W. C., Jr. (1969) *J. Biol. Chem.* **244**, 5695–5702
- Conway, A. & Koshland, D. E., Jr. (1968) *Biochemistry* **7**, 4011–4023
- Cook, B. A. & Koshland, D. E., Jr. (1970) *Biochemistry* **9**, 3337–3342
- Curtis, A. R. & Chance, E. M. (1972) *FEBS Meet. 8th* **25**, 39–57
- Dawson, D. M., Eppenberger, H. M. & Kaplan, N. O. (1965) *Biochem. Biophys. Res. Commun.* **21**, 346–353
- Edsall, J. T. (1953) *Enzymes*, 1st edn., **1**, 634
- Ferdinand, W. (1964) *Biochem. J.* **92**, 578–585
- Guidotti, G. (1967) *J. Biol. Chem.* **242**, 3694–3703
- Guidotti, G., Konigsberg, W. & Craig, L. C. (1963) *Proc. Nat. Acad. Sci. U.S.* **50**, 774–782
- Harrington, W. F. & Karr, G. H. (1965) *J. Mol. Biol.* **13**, 885–893
- Harris, J. I. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), pp. 57–70, Springer-Verlag, Berlin
- Harris, J. I. & Perham, R. N. (1965) *J. Mol. Biol.* **13**, 876–884
- Hoagland, D. B., Jr. & Teller, D. C. (1969) *Biochemistry* **8**, 596–602
- Huehns, E. R., Shooter, E. M. & Beaven, G. H. (1962a) *J. Mol. Biol.* **4**, 323–328
- Huehns, E. R., Dance, N., Shooter, E. M., Beaven, G. H. & Gratzer, W. B. (1962b) *J. Mol. Biol.* **4**, 329–337
- Jaenicke, R. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), pp. 71–90, Springer-Verlag, Berlin
- Jones, G. M. T. & Harris, J. I. (1972) *FEBS Lett.* **22**, 185–189
- Kauzmann, W. (1959) *Advan. Protein Chem.* **15**, 1–63
- Keresztes-Nagy, S., Lazer, L., Klapper, M. H. & Klotz, I. M. (1965) *Science* **150**, 357–359
- Kirschner, K. (1971) *J. Mol. Biol.* **58**, 51–68
- Kirschner, K. & Schuster, I. (1970) in *Pyridine Nucleotide Dependent Dehydrogenases* (Sund, H., ed.), pp. 217–228, Springer-Verlag, Berlin
- Kirschner, K., Gallego, E., Schuster, I. & Goodall, D. (1971) *J. Mol. Biol.* **58**, 29–50
- Kochman, M., Golebiowska, J., Baranowski, T., Dedman, J. R., Fodge, D. W. & Harris, B. G. (1974) *FEBS Lett.* **41**, 104–107
- Krebs, E. (1952) *J. Biol. Chem.* **200**, 479–492
- Lazdunski, M. & Vincent, J.-P. (1972) in *Protein-Protein Interactions* (Joenicke, R. & Helmreich, E., eds.), pp. 129–138, Springer-Verlag, Berlin
- Lebherz, H. G., Savage, B. & Abacherli, E. (1973) *Nature (London) New Biol.* **245**, 269–271
- Markert, C. L. (1963) *Science* **140**, 1329–1330
- Meighen, E. A. & Schachman, H. K. (1970a) *Biochemistry* **9**, 1163–1176
- Meighen, E. A. & Schachman, H. K. (1970b) *Biochemistry* **9**, 1177–1184
- Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol.* **12**, 88–118
- Penhoet, E., Rajkumar, T. & Rutter, W. J. (1966) *Proc. Nat. Acad. Sci. U.S.* **56**, 1275–1282
- Penhoet, E., Kochman, M., Valentine, R. & Rutter, W. J. (1967) *Biochemistry* **6**, 2940–2949
- Perham, R. N. (1969) *Biochem. J.* **111**, 17–21
- Rosemeyer, M. A. & Huehns, E. R. (1967) *J. Mol. Biol.* **25**, 253–273
- Smith, G. D. & Schachman, H. K. (1973) *Biochemistry* **12**, 3789–3801
- Smoluchowski, M. V. (1916) *Phys. Z.* **17**, 557–583
- Smoluchowski, M. V. (1917) *Z. Phys. Chem. Abt. A* **92**, 129–135
- Spotorno, G. L. M. & Hollaway, M. R. (1970) *Nature (London)* **226**, 756–757
- Stancel, G. M. & Deal, W. C., Jr. (1969) *Biochemistry* **8**, 4005–4011
- Trentham, D. R. (1968) *Biochem. J.* **109**, 603–615
- Watson, H. C. & Banaszak, L. J. (1964) *Nature (London)* **204**, 918–920
- Watson, H. C., Duée, E. & Mercer, W. D. (1972) *Nature (London)* **240**, 130–133