Subunit Ratios of Separated Hybrid Hexamers of *Neurospora* NADP-Specific Glutamate Dehydrogenase Containing Complementing Mutationally Modified Monomers

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The am^1 and am^3 mutational variants of the *Neurospora crassa* NADP-specific glutamate dehydrogenase show complementation activity in hybrid hexamers. A freeze-thaw hybridization method was used to construct hybrids from purified enzymes and the products were separated into species of different monomer ratio by affinity chromatography. Hexamers with $am^1:am^3$ ratios of 1:5, 2:4, 3:3, 4:2 and 5:1 were all recovered as resolved or partially resolved peaks in quantities approximating to a binomial distribution. Reassociation of monomers during the hybridization process was random, except for some differential loss of am^3 protein by precipitation and an apparent absence of reassociated am^1 homohexamers. Complementation activity was shown by hybrids of all five monomer ratios, owing to activation of am^3 monomers by conformational constraints arising from the intrinsically inactive am^1 monomers. The activating effect of such constraints was greatest in hexamers containing only a single am^1 monomer and least in the $5am^1:1am^3$ species. When fully activated by L-glutamate all am^3 monomers were equivalent in intrinsic catalytic activity, irrespective of the number of am^1 monomers per hexamer.

The NADP-specific glutamate dehydrogenase of *Neurospora crassa* (EC 1.4.1.4) is a hexameric enzyme with identical polypeptide chains. The amino acid sequence of 452 residues shows clear homology for most of the chain to the well-studied glutamate dehydrogenase of ox liver (Wootton *et al.*, 1974). The mutational variants of this *Neurospora* enzyme, in many of which amino acid substitutions have been defined (Brett *et al.*, 1976; Fincham & Baron, 1977), provide a particularly favourable system for the study of the mechanisms underlying several enzymological and genetic phenomena. We report here part of a study in which hybrid hexamers, made by hybridization *in vitro* of pairs of variants that differ in defined amino acid substitutions, are used to analyse intersubunit interactions and allosteric conformational equilibria.

In earlier work on this system [reviewed by Fincham (1966) and Zabin & Villarejo (1975)] Fincham and colleagues established that genetic complementation between allelic mutants of the am gene (the structural gene encoding NADP-specific glutamate dehydrogenase) was caused by the formation of hybrid oligomers. In complementing hybrid enzymes, which could originate *in vivo* in heterokaryons or be made *in vitro* from purified variant enzymes, intersubunit

interactions caused correction or partial correction of defects in mutationally modified subunits. Evidence for conformational correction was obtained for complementing hybrid enzymes which contained subunits of the am^1 variant as one component and any one of the am^2 , am^3 or am^{19} variants as the other. The am^1 protein (amino acid substitution serine-336 replaced by phenylalanine) is completely inactive owing to a localized defect in coenzyme binding, but is otherwise of almost normal conformation, as shown by the variation of its intrinsic tryptophan fluorescence with changes in pH and dicarboxylate concentration. The enzymes of variants am^2 (histidine-142 replaced by glutamine), am^3 (glutamate-393 replaced by glycine) and am^{19} (lysine-141 replaced by methionine) are all potentially active, but are in different ways defective in an allosteric conformational equilibrium between active and inactive states, being abnormally stable in inactive states. [Brett *et al.* (1976), Ashby *et al.* (1974) and Ashby (1974) give details of amino acid substitutions and interpretation of defects in these variants.] Thus the hypothesis of conformational correction proposes that in hybrid enzymes the inactive subunits of am^1 protein stabilize through intersubunit constraints the subunits of am^2 , am^3 or am^{19} enzymes in their normally inaccessible active conformations.

A long-standing barrier to detailed tests of this hypothesis has been the complexity of the mixture of

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hybrid enzymes, presumed to be of different subunit ratio, formed as the product of hybridization *in vivo* or *in vitro*. The component hybrids failed to separate by conventional techniques of ion-exchange chromatography, centrifugation, gel electrophoresis or isoelectric focusing (Fincham & Coddington, 1963a; Coddington & Fincham, 1965; Coddington *et al.*, 1966; D. H. Watson & J. C. Wootton, unpublished work). This limitation has now been overcome by an affinity-chromatographic system using 8-(6-amino-hexyl)amino-2'-AMP-Sepharose 4B (an immobilized derivative of the NADP analogue 2'-AMP), which separated the mutational variants and appeared to resolve hybridized variants into multiple chromatographically distinct species (Watson & Wootton, 1977). The *am*¹ variant, as expected from its defect in coenzyme binding, showed a very low affinity for this adsorbent. Therefore, from theoretical considerations of mechanisms of affinity chromatography, this adsorbent is expected to separate *am*¹-containing hybrid hexamers into species differing in the number of *am*¹ monomers. We describe here the assignment of monomer ratios to hybrid hexamers of *am*¹ and *am*³ proteins separated by this system, and some enzymological properties of these separated hybrids.

The unseparated product of hybridization of *am*¹ and *am*³ enzymes is of homogeneous molecular weight indistinguishable from the native hexamers (Fincham & Coddington, 1963a,b; Coddington & Fincham, 1965, and confirmed in recent work; J. C. Wootton & A. J. Baron, unpublished work). Separated hybrid enzymes may therefore safely be assumed to be hexameric. In the present paper 'monomer' refers to the polypeptide chain of mol.wt. 48600, and 'hexamer' to the normal native state of the enzyme of mol.wt. 291000. Higher-order polymerization, which has generated confusion in similar terminology applied to ox liver glutamate dehydrogenase, has not been observed in the *Neurospora* enzyme.

Materials and Methods

Materials

Iodo[2-¹⁴C]acetic acid (40–60 μ Ci/ μ mol) and iodo[2-³H]acetic acid (50–150 μ Ci/ μ mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive iodoacetic acid, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Scintillation-grade toluene was obtained from BDH Chemicals, Poole, Dorset, U.K., and NCS tissue solubilizer from Hopkin and Williams, Romford, Essex, U.K. All other materials were as described previously (Ashby *et al.*, 1974; Watson & Wootton, 1977).

Strains of Neurospora crassa, growth of mycelium and enzyme purification

These were as described previously (Ashby *et al.*, 1974; Watson & Wootton, 1977).

Enzyme assays

These were essentially as described by Coddington *et al.* (1966). Assay system A measures the rate of oxidation of NADPH during the reductive amination of 2-oxoglutarate at pH 8.5. Assay system C measures the rate of reduction of NADP⁺ during the oxidative deamination of L-glutamate at pH 8.5. Concentration of L-glutamate in system C was 150 mM unless otherwise stated. Other standard conditions were as described by Watson & Wootton (1977). Enzyme activities (initial velocities) are expressed in units of μ mol of substrate transformed/min.

Enzyme hybridization

Mixtures of purified *am*¹ and *am*³ proteins were hybridized by the method of freezing to -20°C followed by slow thawing (Coddington *et al.*, 1966), in 50 mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 0.1 M-NaCl, by using the technique described by Watson & Wootton (1977). This method gave a greater yield of complementation activity than the method of transient acidification used by Fincham & Coddington (1963a,b).

Partial carboxymethylation of enzyme in the native state

Traces of I₂ were removed from iodoacetic acid by sublimation under vacuum of solutions in water, which were subsequently dried to give white crystalline iodoacetic acid suitable for carboxymethylation reactions. Reaction conditions were as described in Table 1, amounts of non-radioactive, ¹⁴C- or ³H-labelled acid being used as appropriate for different experiments. The pH of the reaction mixture, which was continuously monitored in some experiments, did not alter significantly during the reaction.

Affinity chromatography on 8-(6-amino-hexyl)amino-2'-AMP-Sepharose 4B

The general methods were as described by Watson & Wootton (1977), by using a column of adsorbent of dimensions 0.8 cm $\times$ 8 cm, operated at 6°C. Samples of hybridized enzymes were dialysed into 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA, and were applied to the column, which had been equilibrated with at least 6 bed vol. of the same buffer. After application of the sample the flow was

stopped for 1 h to allow equilibration of enzyme and adsorbent. Elution was initially with at least 3 bed vol. of the above buffer to remove any unbound enzyme, although in the experiments described here no unbound enzyme was detected by activity, A_{280} or radioactivity measurements. Retarded enzyme was then eluted with a linear gradient of 0–2.5 mM-NADP⁺, all in the above buffer. The elution flow rate was 8 ml/h.

Radioactivity measurements

For measurements of ³H or ¹⁴C in singly labelled samples, solutions were freeze-dried in glass scintillation vials. The residue was solubilized, with the vials sealed with Teflon inserts, in 0.22 ml of NCS tissue solubilizer containing 10% (v/v) water per sample, for 16 h at 37°C. The vials were cooled to approx. 21°C and 10 ml of scintillation liquid (toluene containing 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-(5-phenyloxazol-2-yl)benzene/litre) was added to each. Vials were then kept in the dark at 21°C for approx. 24 h before radioactivity counting, to allow for decay of phosphorescence induced in this 'cocktail' by exposure to visible light. Vials were counted at least twice to check that a constant c.p.m. value was thus obtained, in a Beckman LS230 liquid-scintillation counter, and background c.p.m. was subtracted.

For double-labelling experiments, a sufficiently accurate method of determining d.p.m. of ³H and ¹⁴C was based on standardization experiments as follows. Samples of standard singly labelled and standard doubly labelled mixtures of proteins (prepared volumetrically at known ratios) were counted for radioactivity before and after additions to the vials of known amounts of d.p.m. of ³H- and ¹⁴C-labelled hexadecane internal standards. These showed a net counting efficiency of 57.1% in the ¹⁴C channel, an overlap into the ³H channel of 41.0% of the ¹⁴C c.p.m., and a net counting efficiency of 25.9% in the ³H channel. These values were constant at many different concentrations of standard proteins, showing that there was no measurable variation in quenching over the full range of concentrations present in the experimental samples. Also, concentrations in samples of NADP⁺ of up to 2.5 mM were found to cause no detectable colour quenching. Therefore a combined correction for quenching and counting efficiency was made, based on these values, to the ¹⁴C-channel c.p.m. after correction for background, and to the ³H-channel c.p.m. after corrections for background and overlap of ¹⁴C counts. Doubly labelled standard mixtures of proteins, of ¹⁴C/³H ratios chosen to simulate those in experimental samples, showed that, after application of the above corrections, errors in d.p.m. determinations were not appreciably greater than the statistical error in

counting. To minimize the effect of statistical error on overlap of ¹⁴C counts into the ³H channel, proteins for double-labelling experiments were prepared so that the initial d.p.m. of ³H per mg of protein was approx. 10 times that of ¹⁴C (see legend to Fig. 2). In all cases sample preparation and control for phosphorescence were as described for singly labelled samples.

Results and Discussion

Labelling of proteins by partial carboxymethylation

A convenient analytical technique has not yet been found for banding in gels *am*¹–*am*³ hybrid hexamers of different subunit ratio. Therefore a double-labelling strategy was used to quantify and assign subunit ratios to the hybrid components separated by affinity chromatography. The possibility of carboxymethylation to introduce radioactive label into the native state of the enzyme was suggested by the claim (Blumenthal & Smith, 1973) that the wild-type enzyme could be treated with a large excess of iodoacetate at pH 8.3 with no loss of enzymic activity.

Under conditions of a tenfold molar excess of iodoacetate over protein thiol groups at pH 8.0, approx. 2 thiol groups per monomer of wild-type, *am*¹ and *am*³ enzymes were carboxymethylated (Table 1). The time course of incorporation of ³H and ¹⁴C from radioactive iodoacetate into protein was followed under these conditions and a plateau was approached after 2 h incubation at 21°C. At 2 h the amount of radioactivity incorporated was equivalent to between 2 and 3 carboxymethyl groups bound per monomer, in agreement with the decreased number of thiol groups available to 5,5'-dithiobis-(2-nitrobenzoic acid) (Table 1).

No change in enzymic activity in either direction or in the effect of temperature on activity was caused by this modification (Table 1). For both wild-type and *am*³ enzymes, native and partially carboxymethylated proteins showed identical elution positions in chromatography under standard conditions on the 8-(6-aminohexyl)amino-2'-AMP-Sepharose 4B affinity adsorbent (elution positions of the native proteins are given by Watson & Wootton, 1977). Hybridized *am*¹ and *am*³ enzymes gave almost indistinguishable profiles of multiple peaks in chromatography on this adsorbent in cases where the proteins used for hybridization were both unmodified (Fig. 1a), both partially carboxymethylated (Figs. 2a and 2b), or the *am*¹ component unmodified and the *am*³ component partially carboxymethylated (Fig. 1b). This shows that the pattern of subunit association is not affected by the presence of carboxymethyl groups. Thus this modification is a suitably mild method of introducing radioactive label into *Neurospora* NADP-specific glutamate dehydrogenase.

Table 1. *Partial carboxymethylation of wild-type, am¹ and am³ proteins and lack of effect on enzymic activity*
Quantities of a solution of iodoacetic acid in 1 M-NaOH were added to enzyme solutions in 0.05 M-sodium phosphate buffer, pH 8.0, to give a molar concentration of iodoacetate 10 times that of cysteine residues per monomer (based on six cysteine residues per monomer of mol.wt. 48 500). After 2 h incubation at 21°C the solutions of partially carboxymethylated enzymes were dialysed against a large excess of 0.05 M-sodium phosphate buffer, pH 8.0. Samples of the modified enzymes (after dialysis) and unmodified enzymes were made 2% (w/v) in sodium dodecyl sulphate for determination of total thiol groups by titration with 5,5'-dithiobis-(2-nitrobenzoic acid) by the method of Habeeb (1972). Assay systems A and C were as described in the Materials and Methods section. $v(36^\circ\text{C})/v(26^\circ\text{C})$ is the ratio of initial velocities at 36 and 26°C under otherwise standard assay system C conditions.

Protein	Number of thiol groups per monomer	Number of thiol groups modified per monomer	Specific activity (units/mg of protein)		$\frac{v(36^\circ\text{C})}{v(26^\circ\text{C})}$
			Assay system A	Assay system C	
Wild-type (native)	5.8	—	295	98	2.0
Wild-type (carboxymethylated)	4.4	1.6	293	98	2.0
am ¹ (native)	6.7	—	0	0	—
am ¹ (carboxymethylated)	4.3	1.7	0	0	—
am ³ (native)	6.1	—	0	42	11.1
am ³ (carboxymethylated)	4.0	2.0	0	47	11.0

Fig. 1. *Affinity chromatography on 8-(6-aminoethyl)amino-2'-AMP-Sepharose 4B of hybrid hexamers derived from hybridized equimolar mixtures of am¹ and am³ proteins: lack of effect of partial carboxymethylation*

Hybridized enzyme was made by the freeze-thaw procedure, described in the Materials and Methods section, from equimolar mixtures of native am¹ and am³ proteins (a), or native am¹ protein and partially carboxymethylated am³ protein (b) (modified by reaction with iodo[³H]acetate by the method described in Table 1). The product was centrifuged (10 min at 8000g) and the supernatant was dialysed against 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA, applied to the column of affinity adsorbent, and eluted under standard conditions (see the Materials and Methods section). The straight line on each graph indicates the linear gradient from 0 to 2.5 mM-NADP⁺, calculated as the concentration in the eluted fractions. Fraction volume was 1.62 ml. In (a) enzymic activity was measured in assay system C containing 300 mM-L-glutamate (○). In (b) ³H radioactivity was measured (average of two determinations is plotted, □). Both enzymic and radioactivity measurements are proportional to the amount of the am³ component in each fraction (see the text).

Instability of am^3 subunits during hybridization

In hybridization experiments involving equimolar mixtures of am^1 and am^3 proteins, about half the am^3 component, but almost none of the am^1 component, was lost in a precipitate that formed during the freeze-thaw procedure. In a hybridization using [3H]carboxymethylated am^3 protein and unmodified am^1 protein only 49% (5580 c.p.m.) of the input of 3H (11400 c.p.m.) was recovered in solution after freeze-thaw treatment, dialysis and centrifugation. The sedimented precipitate accounted for the remaining half of the input c.p.m. In the experiment shown in Fig. 2, ^{14}C -labelled am^1 protein was effectively completely recovered in hybrid hexamers after affinity chromatography (4900 d.p.m. recovered out of 5400 d.p.m. input, the 10% loss being residual volume not recovered from dialysis). In contrast approx. 60% loss of the 3H -labelled am^3 protein occurred in addition to the 10% volume loss (18580 d.p.m. recovered out of 58420 d.p.m. input), this being attributable to precipitation during the freeze-thaw treatment. Control experiments showed that no measurable loss of 3H was caused by lability or exchangeability of the 3H atoms of the labelled carboxymethyl groups. This effect was not an artifact, since the c.p.m. of am^3 protein carboxymethylated with iodo[3H]acetate was stable to prolonged dialysis, and in the experiment shown in Fig. 1(b) there was almost complete recovery in the eluted fractions of the soluble 3H -labelled material applied to the column. These controls show that it is valid to calculate the relative amounts of am^1 and am^3 subunits in separated hybrid hexamers from the original specific radioactivities (corrected to d.p.m.) of the am^1 and am^3 proteins measured before hybridization.

Subunit compositions of chromatographically distinct hybrid hexamers

The pattern of multiple peaks obtained from affinity chromatography of hybridized equimolar mixtures of am^1 and am^3 proteins was very reproducible (Figs. 1a, 1b, 2a and five other almost identical runs not shown). The peaks represent chromatographically distinct entities. Each peak was eluted at its original position in the $NADP^+$ gradient on rechromatography of pooled fractions after $(NH_4)_2SO_4$ precipitation, redissolving and dialysis. Rechromatographed peaks were very narrow, typically equivalent to two or three fractions of the profile shown in Fig. 2(a), resembling rechromatographed peaks in Fig. 7 of Watson & Wootton (1977). Preliminary assays showed that the peaks differed quantitatively in the extent of activation by high concentrations of L-glutamate, suggesting that they represented resolved or partially resolved species of hybrid hexamer differing in subunit ratio.

The subunit compositions of these peaks were determined directly in the double-labelling experiment shown in Fig. 2. The amounts of am^1 and am^3 proteins in each fraction of the profile of activity peaks (Fig. 2a) were calculated from the d.p.m. of respectively ^{14}C and 3H . These amounts were summed to give total protein (Fig. 2b). The proportions of am^1 and am^3 proteins, when expressed as number of am^1 monomers per hexamer (Fig. 2c), showed good agreement within experimental error to whole-number values for almost all the fractions. Fractions 21, 30 and 39 are subject to relatively large experimental error because of the small amounts of protein present. For all other fractions the points may be considered estimates of mean values whose standard errors are less than the diameter of the closed circles in Fig. 2(c).

This agreement with whole-number values suggests that each fraction except no. 25 contains predominantly one species of hybrid hexamer of a single ratio of monomers. The narrowness of the peaks, which is retained on rechromatography, is also consistent with the conclusion that the incompletely resolved hybrid species show little overlap. The data suggest the following assignments of monomer ratio: fraction 21, $1am^1:5am^3$; fractions 23 and 24, $4am^1:2am^3$; fraction 25, mixture of $4am^1:2am^3$ and $2am^1:4am^3$; fractions 26 to 28, $2am^1:4am^3$; fraction 29, $3am^1:3am^3$; fraction 31, $4am^1:2am^3$; fractions 32–39, $5am^1:1am^3$. Independent evidence from measurements of specific enzyme activity (discussed below in the section on activity of individual monomers) supports these assignments.

The peaks of hybrid hexamers were eluted in order of increasing content of am^1 monomers, except for the unexpected duplication of peaks of ratio $4am^1:2am^3$, which is not understood. It might reflect the unexplained heterogeneity in elution position shown by unhybridized am^1 homohexamers (Watson & Wootton, 1977), but this seems unlikely because the am^1 – am^3 hybrid hexamers of other subunit ratios appeared as single peaks. Alternatively there may be differences in chromatographic properties between different 'geometric isomers' of hexamers of ratio $4am^1:2am^3$. ['Geometric isomers' are topologically non-equivalent oligomeric configurations of the same ratio of two types of monomer. For example, for hybrid hexamers of maximum symmetry (32 or D_3 point group symmetry, except for detailed differences between the two types of almost-identical monomer) four geometric isomers are possible for each of the 2:4, 3:3 and 4:2 monomer ratios.]

Activity of individual monomers in hybrid hexamers

L-Glutamate acts as both substrate and activator of unhybridized am^3 homohexamers [see Fig. 5(c) of Watson & Wootton (1977) for the sigmoid dependence

Fig. 2. Chromatography on 8-(6-aminohexyl)amino-2'-AMP-Sepharose 4B of doubly labelled am^1 - am^3 hybrid hexamers and assignment of subunit compositions

Hybridized enzyme was made by the freeze-thaw procedure (described in the Materials and Methods section) from 0.4 mg of partially carboxymethylated am^1 protein (specific radioactivity 1.35×10^4 d.p.m. of ^{14}C /mg) and 0.4 mg of partially carboxymethylated am^3 protein (specific radioactivity 1.46×10^5 d.p.m. of ^3H /mg). Correction of c.p.m. to d.p.m. is described in the Materials and Methods section. After dialysis against 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA, 0.5 ml of the dialysis residue (which contained a slight precipitate), containing approx. 90% of the original radioactivity, was applied to the 8-(6-aminohexyl)amino-2'-AMP-Sepharose 4B column and eluted under standard conditions (see the Materials and Methods section). The straight lines in (a) and (b) indicate the linear gradient of NADP⁺ concentration in the eluted fractions from 0 in fraction 13 to 2.5 mM in fraction 44. Fraction volume was 1.62 ml. (a) Enzymic activity (total units in each fraction) in assay system C containing 300 mM-L-glutamate (○). (b) Total protein (□) calculated from the d.p.m. of ^{14}C and ^3H in each fraction (each divided by the original specific radioactivities before hybridization of the am^1 and am^3 components respectively). (c) Average number of am^1 monomers per hexamer for each fraction (●), calculated as $6 \times$ the amount of am^1 component divided by the total protein shown in (b). In (c) the horizontal lines indicate the whole-number values of am^1 monomers per hexamer, and the points in

of am^3 enzyme activity on L-glutamate concentration]; 0.3 M-L-glutamate is effectively saturating in assay-system-C conditions, giving a specific activity of 60 units/mg of am^3 enzyme (mean of many measurements). Under these conditions the am^3 monomers of am^1 - am^3 hybrid hexamers are expected to be fully activated by L-glutamate, irrespective of any activating constraints that may arise from am^1 monomers. Since am^1 protein is intrinsically inactive, the specific activity of hybrid hexamers in this assay is expected to be proportional to the number of am^3 monomers per hexamer, provided that this activity is an intrinsic property of am^3 monomers.

This expectation is borne out by the data in Fig. 3, in which specific-activity measurements of the fractions of Fig. 2 (0.3 M-L-glutamate assay) are plotted in groups according to the monomer ratios assigned from the double-labelling experiment. For all the species of hybrid hexamer, each am^3 monomer contributed a specific enzymic activity of 10 units/mg of hexamer. Therefore am^3 monomers, when effectively fully activated in the 0.3 M-L-glutamate assay, showed a characteristic catalytic-centre activity (485 molecules of substrate transformed/min per catalytic centre) which was apparently unaffected by interactions with am^1 monomers. This constancy of am^3 catalytic-centre activity at all monomer ratios shows that monomer compositions of am^1 - am^3 hybrid hexamers may be reliably determined from measurements of specific activities in this assay.

These results demonstrate that in am^3 homo-hexamers that are fully activated by L-glutamate all six monomers are active and equivalent. The wild-type enzyme gives a somewhat higher catalytic-centre activity in the 0.3 M-L-glutamate assay of approx. 790 molecules of substrate transformed/min per monomer. These experiments provide evidence against the occurrence of 'half-of-the-sites reactivity' in *Neurospora* NADP-specific glutamate dehydrogenase, at least when the enzyme is effectively saturated with L-glutamate.

Complementation activity in am^1 - am^3 hybrid hexamers

Complementation activity may be regarded as enzymic activity given by am^1 - am^3 hybrid hexamers under conditions in which am^3 homo-hexamers give

parenteses are subject to a relatively large experimental error because of the small amount of protein present. Recoveries (d.p.m. summed over the column fractions/d.p.m. in the 0.5 ml of dialysed hybridization mixture applied) were: am^1 protein, 102%; am^3 protein, 44%. Loss of am^3 protein is attributable to the precipitate, formed during hybridization, which was not removed by centrifugation in this experiment and was not eluted from the column.

Fig. 3. Specific activity with 0.3 M-L-glutamate of am^1 - am^3 hybrid hexamers of different monomer ratio

Numbers of am^3 monomers per hexamer are assigned from the double-labelling experiment of Fig. 2(c). Specific enzymic activity ($\circ$) is calculated from the data on activity in assay system C containing 0.3 M-L-glutamate (Fig. 2a) and amount of protein (Fig. 2b). Each point refers to a different fraction of the chromatographic profile shown in Fig. 2, except for the case of six am^3 monomers per hexamer, where the point is the mean of four measurements of specific activity of purified am^3 enzyme.

significantly less activity or no activity. Such complementation reflects activation of am^3 monomers by intermonomer conformational interactions involving the intrinsically inactive but conformationally normal am^1 monomers. A sensitive indication of the occurrence of intersubunit interactions is given by comparisons of the catalytic-centre activity per am^3 monomer in am^3 homohexamers and in hybrid enzymes of defined monomer ratio. In contrast with

the fully activating conditions discussed above, at concentrations of L-glutamate below about 150 mM activation of am^3 homohexamers by L-glutamate alone is only partial (see Fig. 5c of Watson & Wootton, 1977). In Table 2, specific-activity measurements at 150 mM- and 75 mM-L-glutamate of the different am^1 - am^3 hybrid hexamers and am^3 homohexamers are expressed as catalytic-centre activity per am^3 monomer.

At 75 mM-L-glutamate, strong activation of am^3 monomers by am^1 monomers occurred in hybrid hexamers of all monomer ratios (Table 2). The activation was greatest (approx. 5-fold) for 1 am^1 :5 am^3 hexamers, least (approx. 2-fold) for 5 am^1 :1 am^3 hexamers, and approx. 3.5-fold for the three intermediate species of hybrid hexamers. At 150 mM-L-glutamate activation of am^3 monomers by the single am^1 monomer was apparent for the 1 am^1 :5 am^3 species, and there was an indication of inhibition of activity of the single am^3 monomer in the 5 am^1 :1 am^3 species (Table 2).

In general, these results support the hypothesis that am^3 monomers are stabilized in an active conformation by constraints arising from interactions with am^1 monomers. This hypothesis is also substantiated by direct observations of the conformational states of the am^1 and am^3 homohexamers and the separated hybrid hexamers from the intrinsic tryptophan fluorescence of these proteins (B. Ashby, D. H. Watson & J. C. Wootton, unpublished work). However, the behaviour of 1 am^1 :5 am^3 hexamers is unexpected. This species resembles am^3 homohexamers in showing very low activity at 26°C (Table 2). The other four species of hybrid hexamer resemble the wild-type enzyme in showing a 2-fold difference in activity between 26 and 36°C (Table 2). The 1 am^1 :5 am^3 species, however, shows surprisingly high catalytic-centre activity per am^3 monomer. Clearly the presence of only a single am^1 monomer in this hybrid hexamer is sufficient to cause considerable stabilization of the active conformation of the five am^3 monomers. The presence of further am^1

Table 2. Some enzymological properties of am^1 - am^3 hybrid hexamers of different monomer ratios

Assays were carried out on unhybridized am^3 homohexamers and on samples of fractions from a chromatographic separation, similar to that shown in Fig. 1(a), of a hybridized equimolar mixture of native am^1 and am^3 proteins. Monomer ratios were assigned by comparison with the experiment shown in Fig. 2, and amounts of protein were calculated from assays in system C containing 0.3 M-L-glutamate by using the specific-activity values of the experiments shown in Figs. 2 and 3. Results of system-C assays containing the stated concentrations of L-glutamate were averaged for all fractions of the same monomer ratio and used for calculations of catalytic-centre activity per am^3 monomer. $v(36^\circ\text{C})/v(26^\circ\text{C})$ is the ratio of initial velocities at 36°C and 26°C under otherwise standard assay-system-C conditions. This ratio is 2.0 for the wild-type enzyme.

		No. of am^3 monomers per hexamer					
		1	2	3	4	5	6
Catalytic-centre activity (molecules of substrate transformed/min per am^3 monomer)	75 mM-L-Glutamate	140	230	220	240	340	65
	150 mM-L-Glutamate	220	340	290	270	450	300
	150 mM-L-Glutamate	2.1	2.2	2.1	2.0	11.1	11.1

monomers in the other four species of hybrid hexamers is apparently detrimental to this strong activation, especially in the $5 am^1:1 am^3$ species.

This phenomenon may be explained by either or both of two possibilities. Firstly, the free energy of interaction of am^1 monomers with their neighbouring monomers may be more tightly coupled to conformational equilibria in other am^1 monomers that are irrelevant to enzymic activity than to the relevant equilibria in am^3 monomers that involve the active state. Secondly, the activating conformational equilibria in am^3 monomers may be linked allosterically to NADP⁺ binding by neighbouring monomers. Since am^1 monomers fail to bind coenzyme, any coenzyme-linked co-operative activating effects would decrease with increasing content of am^1 monomers per hexamer. Further work is required to explore these possibilities in detail.

Random reassortment of monomers during hybridization

Random reassortment of monomers of an equimolar hybridization mixture is expected to yield a symmetrical binomial distribution of species of all possible monomer ratios. The distribution observed (Figs. 1 and 2) is not symmetrical, since there is a clear excess of the $4 am^1:2 am^3$ and $5 am^1:1 am^3$ species. However, if allowance is made for the observed loss of am^3 subunits during the freeze-thaw hybridization procedure, the fit to a binomial distribution becomes good. The average proportions of the am^1 and am^3 components recovered in re-associated hexamers from six separate runs (those shown in Figs. 1 and 2 and three others of very similar chromatographic profile) were 0.70 of am^1 (range 0.64–0.73) to 0.30 of am^3 . For the runs in which radioactive label was not used, the relative amounts of protein in each of the five hybrid species was calculated from measurements of specific enzymic activity in the assay with 0.3M-L-glutamate by using monomer ratios assigned by comparison with Fig. 2. These proportions were not significantly affected by partial carboxymethylation. Therefore the expected binomial distribution was calculated from a probability of 0.70 of obtaining am^1 monomers.

Fig. 4 shows the distribution of hybrid hexamers observed in the experiments of Figs. 1(a), 1(b) and 2 compared with this expected binomial distribution. The discrepancies may be explained by experimental variation, except for an absence of am^1 homohexamers and an excess of the $5 am^1:1 am^3$ species. At the protein concentrations used in the experiment of Fig. 2, am^1 homohexamers would be readily detectable, since they would be largely unretarded by the affinity adsorbent (Watson & Wootton, 1977). However, there was no detectable protein at the expected elution position of am^1 protein at approxi-

Fig. 4. Observed and theoretical distributions of the amount of protein in each of the seven possible species of different ratios of am^1 and am^3 monomers

The amount of protein recovered in each species of hybrid hexamer is calculated from the data of Fig. 1(a) (bars labelled A), Fig. 1(b) (bars labelled B) and Fig. 2(b) (bars labelled C), by using assignments of monomer ratios deduced from the experiment shown in Fig. 2. For the data from Fig. 1, total protein is calculated from the amount of am^3 component (proportional to enzyme activity in Fig. 1a and to 3H radioactivity in Fig. 1b) multiplied by a factor appropriate to the monomer ratio assigned. Black bars labelled X show the theoretical binomial distribution calculated from a probability of recovering am^1 monomers of 0.70. This probability is based on the observed proportions in the recovered protein (mean of six experiments) of 0.70 of am^1 to 0.30 of am^3 .

mately fraction 10 of Fig. 2(b). The expected very small quantity of am^3 homohexamers, which would be eluted at approximately fraction 25 of Fig. 2 (see also Fig. 2 of Watson & Wootton, 1977), would not be detectable in these experiments. The deficiency of am^1 homohexamers is not understood. It is possible that deviations from randomization of monomers may be caused by differences in stability of the contacts between equivalent am^1 – am^1 , am^1 – am^3 and am^3 – am^3 intermonomer interfaces.

Similar experiments have been carried out on hybrid hexamers made from am^1 protein and a different, potentially active mutational variant, am^{19} . These am^1 – am^{19} hybrids contrasted with am^1 – am^3 hybrids in showing considerable deviations from random reassociation, with a strongly preferential recovery of the $2 am^1:4 am^{19}$ and $4 am^1:2 am^{19}$ species (D. H. Watson & J. C. Wootton, unpublished work). This suggests that am^{19} protein forms stable homodimer intermediates which may either fail to dissociate or may preferentially re-form in the freeze-thaw procedure. For am^1 – am^3 hybrid hexamers the presence, in approximately binomial proportions, of all five hybrid species demonstrates that during the freeze-thaw procedure the am^1 and am^3 proteins either dissociate completely to single polypeptides or form structures that freely exchange single polypeptides.

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