ALTERED PROPERTIES OF THE ENZYME, ADENYLOSUCCINASE, PRODUCED BY INTERALLELIC COMPLEMENTATION AT THE AD-4 LOCUS IN NEUROSPORA CRASSA*

C. W. H. Partridge

Department of Botany, Josiah Willard Gibbs Research Laboratories, Yale University, New Haven, Connecticut.

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Studies on interallelic complementation at the ad-4 (adenylosuccinase-less) locus in Neurospora were summarized in 1958 (Woodward, Partridge, and Giles, 1958) and extended in the following year (Woodward, 1959). The enzyme adenylosuccinase catalyzes the splitting of fumarate from adenosine monophosphate succinate (AMP-S) as well as from an analogous precursor, SAICAR (N-(5-amino-1-ribosyl-4-imidazole-carbonyl)-L-aspartic acid 5'-phosphate). Heterocaryons composed of certain mutants (which, when grown separately, lacked the ability to produce adenylosuccinase activity toward either of the two substrates) were found capable of forming active enzyme. Formation of active enzyme in vitro, with mixed homogenates of separately grown allelic mutants, was also observed (Woodward, 1959); this required mixing freshly prepared ice-cold homogenates for a few minutes before extraction of the enzyme at 4°C. Particular pairs of allelic mutants were found to give results, positive or negative, in vitro which were in agreement with the in vivo behavior of the same pairs. In contrast to cases of interlocal complementation, interallelic (intralocal) complementation at the ad-4 locus appears to be limited in enzyme yield to about 25% of the wild type level, dropping to much lower values when the alleles are adjacent on the complementation map. Initially it seemed that these observations as a whole could be most simply interpreted by a mechanism in-

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volving the interaction between mutant enzyme molecules in linear form, analogous to recombination at the chromosome level (Woodward et al., 1958). This scheme predicted that in all cases a completely normal enzyme should be produced, along with an equivalent amount of doubly defective enzyme. However, in the further investigations reported below, partially purified adenylosuccinase from two of the heterocaryons examined proved to be much more thermolabile than the wild type enzyme prepared in the same way. Certain other abnormalities, e.g., in reaction to metal salts, to urea and to pH, were also observed in these cases. Although the tests applied to enzymes from two other heterocaryons failed to reveal any significant abnormalities, the question remains open whether any interallelic heterocaryons produce completely normal enzyme, especially in view of Fincham's finding (1959) of abnormalities in the glutamic dehydrogenase produced by heterocaryons of allelic am mutants and of preliminary results with numerous additional ad-4 heterocaryons (cited below).

Materials and methods. Data on the origin and other characteristics of the ad-4 mutants employed for production of partially purified enzymes have been presented previously (Woodward et al., 1958).

The following general procedure was employed in preparing adenylosuccinase: after cell breakage of the washed mycelium by various mechanical means, it was extracted on a shaker at 4°C. for 1 hour with 0.05 M Tris HCl, pH 8.0, containing 3×10^{-3} M mercaptoethanol. After removal of insoluble cell debris by centrifugation (followed in some cases by a second centrifugation in the presence of 0.01 M $MnCl_2$), the extract was chromatographed twice on DEAE-cellulose to remove fumarase and the bulk of nonspecific impurities. The adenylosuccinase activity was recovered from the first column by elution with 0.35 M KCl after prewashing with 0.10 M KCl, and from the second column by gradient elution with 0.05 M to 0.30 M KCl. All eluants also contained the solutes used in extraction.

Assays were performed by continuously recording the change in absorbance at 280 mμ as AMP-S (adenylosuccinic acid) was converted to AMP and

fumaric acid at 35.5° C. in 0.05 M Tris HCl, usually at pH 8.6. Activities were calculated from the linear portions of the curves. Preincubation was carried out at elevated temperatures (in thermolability studies) or at assay temperature (in inhibition studies) in 7/8 of the final assay volume produced by addition of substrate. The AMP-S was generously supplied by Dr. C. E. Carter. The SAICAR, employed only in the case noted in Table I, was the generous gift of Dr. J. M. Buchanan.

Results. Partially purified adenylosuccinase was obtained from four different heterocaryons growing without exogenous adenine. The heterocaryons were formed by cytoplasmic fusion, in pairwise combinations, of six different ad-4 mutants, of various mutagenic origins, located in various parts of the complementation map of the locus. Certain properties of these enzyme preparations (sensitivity, specificity and pH effect) were compared with those of the wild type enzyme with the results summarized in Table I. Further data on thermolability are presented in the figures and in Table II. The procedures employed differed in de-

Table I. Properties of adenylosuccinase produced by ad-4 heterocaryons compared with corresponding properties of wild type enzyme (in parenthesis) in same experiment.

Experiment no.	1	2	3	
Mutant no.	F4 + F23	F14 + F48	F23 + F39	F45 + F48
Thermolability (half-life)	-	10 min. (vs. 13 hrs.)	4 min. (vs. 35 min.)	35 min. (vs. 35 min.)
Metal Inhibition				
10^{-4} M $ZnCl_2$	90%(vs. 90%)	-	60%(vs. 0%)	0%(vs. 0%)
10^{-4} M $CuCl_2$	25%(vs. 25%)	-	60%(vs. 0%)	0%(vs. 0%)
Urea Inhibition (1M)	-	-	100%(vs. 40%)	40%(vs. 40%)
Substrate specificity*	1.0(vs. 1.0)	-	-	-
pH of half maximum activity	-	7.45 (vs. 7.60)	-	-

*Ratio of relative activity (as % wild type activity) on SAICAR to that on AMP-S.

tails in the three experiments. However, within each experiment the heterocaryon enzymes were prepared and treated in the same way as the wild type enzyme included in the same experiment for comparison. Marked abnormalities in thermolability were observed in two cases. In the case of the F 14 + F 48 enzyme the response of activity to pH, while parallel to that of the wild control, reached its half maximal point 0.15 pH unit lower than the wild type value of 7.60. The enzymes obtained from the other two hetercaryons were not found to differ significantly from wild type by the tests listed in Table I. The results summarized in figure 2 and Table II do not indicate that mixing of wild type and thermolabile heterocaryon enzymes either increases the stability of the abnormal enzymes or decreases that of the normal enzyme. Therefore,

Table II. Decay of adenylosuccinase activity with time at 44.5°C. Preincubation at sample dilution of 1:16 in Tris HCl buffer, pH 8.6, 0.05 M, plus 7.5×10^{-3} M mercaptoethanol.

Expt.*	Time (min.)	Wild Type** 74A	Heterocar.** F14 + F48	Mixture Calc.**	Mixture Obs.
2a	0	0.86	0.33	1.19	1.14
	3	-	0.27	1.09	1.09
	8	-	0.20	0.98	0.95
	10	0.78	-	-	-
	15	-	0.12	0.90	0.93
	20	0.78	0.09	0.87	0.88
	30	-	0.04	0.82	0.84
	60	0.74	-	0.78	0.80
2b	0	0.66	0.86	1.52	1.22
	3	0.59	0.66	1.25	1.06
	8	0.54	0.48	1.02	0.92
	15	-	0.30	0.84	0.77
	20	0.53	-	-	-
	25	-	0.15	0.68	0.70
	30	-	0.09	-	-
	35	-	-	0.56	0.59
	40	-	0.06	-	-
	60	0.51	-	0.54	0.56

*Enzyme concentrates from the separate strains and mixtures of them were tested simultaneously in each experiment.
Expt. 2a: mixture of 3 vol. 74A : 7 vol. heterocaryon.
Expt. 2b: mixture of 1 vol. 74A : 9 vol. heterocaryon.
**Relative activities, corrected for proportional content in mixtures.

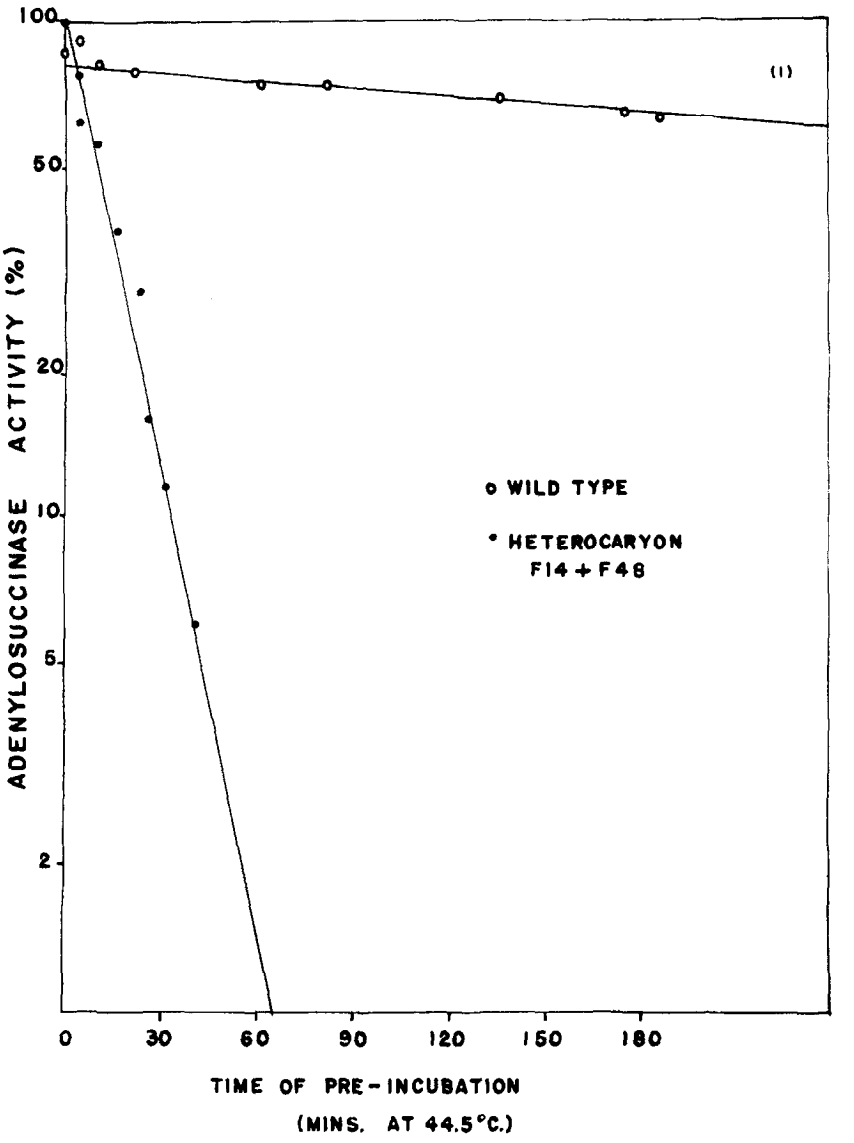

Fig. 1 Decay of adenylosuccinase activity with time at 44.5°C. Preincubation at sample dilution of 1:16 in Tris HCl buffer, pH 8.6, 0.05 M, plus 7.5×10^{-3} M mercaptoethanol.

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the enzyme itself is presumed to be intrinsically different from its wild analog in at least some complementing heterocaryons of ad-4 mutants.

Surveys of crude extracts of a variety of other heterocaryons at the ad-4 locus have given some indication of the frequency of abnormalities in the adenylosuccinase produced. In general, certain alleles tend to confer

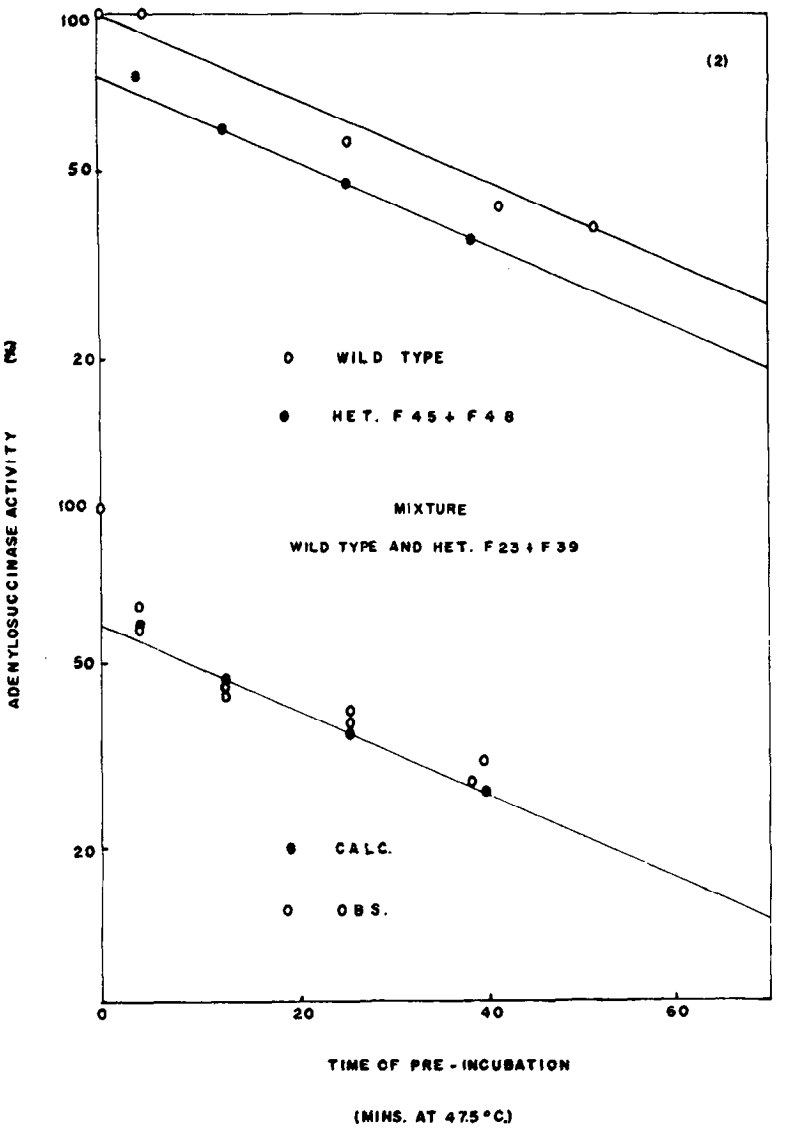

Fig. 2 Decay of adenylosuccinase activity with time at 47.5°C. Preincubation at sample dilution of 1:4 in Tris HCl buffer, pH 8.8, 0.05 M, plus 7.5 x 10^{-3} M mercaptoethanol. Upper half: wild type and heterocaryon F45 + F 48 separately. Lower half: obs.: mixtures of 3 vol. heterocaryon F23 + F39 with 1 vol. wild type; calc: calculated activity of wild type component of mixtures. (No activity from F23 + F39 survived at 4 minutes.)

on the heterocaryons in which they are included, characteristic variations in enzyme yield or behavior in assay. Such properties include a downward shift in pH optimum, thermolability, and sensitivity to mercaptoethanol or other additive. Those alleles showing apparently normal be-

havior and relatively high yield of enzyme activity in heterocaryons with each other are: 103*, 92*, 82*, and 48. Among those behaving abnormally in one or more respects with one or more alleles are: 39, 57*, 58*, 65*, 76*, 89*, 99*, (thermolabile activity); 58*, 89*, (instability toward 0.01 M $MnCl_2$); 45 (instability toward 0.05 M Tris HCl); 45 (inhibition by 0.2 M KCl); 45 (inhibition by low pH); 23, 85*, 86*, 90* (inhibition by high pH); 23, 45, 86* (sensitivity to 3×10^{-2} M mercaptoethanol).

Discussion. It seems clear at this point that interallelic complementation can produce active enzymes of widely different stabilities in different pairs of inactive mutants. Therefore, in its simplest form, a mechanism of dissociation and reciprocal recombination of monomeric enzyme molecules (Woodward et al., 1958) can no longer be entertained either as a general explanation for interallelic complementation or for the ad-4 locus as a whole. A mechanism of this type, for the special case of an enzyme functioning as a polymer, has been suggested by Brenner (1959) and elaborated by Crick and Orgel (1960). It is possible that further studies may provide a basis for a combinational hypothesis which will reduce essentially to the earlier recombinational one (Woodward et al., 1958) in special cases. Further variations in mechanism of interaction are conceivable and cannot yet be ruled out.

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*Alleles of chemical mutagenic origin recently isolated by Dr. Mary Case.