Complementation *in vitro* Between *guaB* Mutants of *Escherichia coli* K12

By H. J. GILBERT AND W. T. DRABBLE*

Department of Biochemistry, University of Southampton, Southampton SO9 3TU

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Guanine auxotrophs of *Escherichia coli* were isolated following mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine or ethyl methanesulphonate. The mutants were classified according to growth properties and absence of IMP dehydrogenase or GMP synthetase activity. Mutations in *guaB* (IMP dehydrogenase-less) were analysed by reversion and suppression tests; all were of the base substitution missense type except for one possible frameshift and one polar nonsense mutation. *GuaB* mutants were examined for protein (CRM) that cross-reacts with monospecific antibodies to IMP dehydrogenase; approximately half were CRM⁺. Enzyme complementation *in vitro* was detected in mixed denatured and renatured cell-free extracts of any CRM⁺ *guaB* mutant and PL1138 (*guaB105*, CRM⁺); CRM⁻ mutants did not complement. *GuaB105* maps distal to all other *guaB* mutations except *guaB86* (CRM⁻). Two hybrid enzymes produced by complementation were less stable to heat than native IMP dehydrogenase, although kinetic constants were similar. These observations indicate interallelic complementation between *guaB* mutants and are consistent with the demonstration of identical subunits for IMP dehydrogenase (Gilbert *et al.*, 1979). Only the subunits supplied by PL1138 are catalytically active in the hybrid enzymes suggesting that this mutant may produce a repairable polypeptide whereas the enzymes of complementing mutants may be defective at the active site.

INTRODUCTION

The biosynthesis of guanosine 5'-monophosphate (GMP) from inosine 5'-monophosphate (IMP) involves two enzymes. IMP dehydrogenase catalyses the conversion of IMP to xanthosine 5'-monophosphate (XMP) and GMP synthetase the conversion of XMP to GMP. IMP dehydrogenase and GMP synthetase are the products of the two structural genes, *guaB* and *guaA* respectively, of the *gua* operon (Lambden & Drabble, 1973). As IMP dehydrogenase catalyses the first reaction unique to guanine nucleotide biosynthesis it plays an important role in controlling the production of GMP by feedback inhibition and by enzyme repression (Mager & Magasanik, 1960; Nijkamp & DeHaan, 1967). The enzyme from *Escherichia coli* has been studied in some detail and this has resulted in conflicting reports about its subunit structure. Krishnaiiah (1975) and Gilbert *et al.* (1978, 1979) have proposed identical subunits whereas Powell (1973) suggested non-identical subunits.

Complementation, the restoration of biological activity by the non-covalent interaction of different polypeptides, can be used to determine the nature of gene products, in particular the number of subunit types in oligomeric proteins. Complementation can be either intercistronic or interallelic (intracistronic). The former involves the interaction of distinct polypeptide chains each specified by its own cistron, as for example, with tryptophan synthetase of *E. coli*. The latter involves interactions between polypeptides from the same cistron and has been demonstrated for several enzymes including alkaline phosphatase and glutamate dehydrogenase (for a general review, see Zabin & Villarejo, 1975).

This report describes the isolation and characterization of *guaB* (IMP dehydrogenase-

less) mutants of *E. coli*. Complementation *in vitro* between the inactive enzymes of these strains is consistent with the presence of identical subunits in IMP dehydrogenase.

METHODS

Bacterial strains. *Escherichia coli* K12 strain W3110 was the prototrophic parental strain of the *gua* mutants listed in Table 1.

Bacteriophage. Phage P1kc was used for transduction as described by Lambden & Drabble (1973).

Media. Defined salts medium (minimal medium) was that of Vogel & Bonner (1956), containing glucose (final concentration 2 mg ml⁻¹) as carbon source. Minimal agar medium was prepared with 1.5% (w/v) Bacto-agar (Difco). Purines, when required, were added to media at a final concentration of 20 µg ml⁻¹. Thiamin was used at a final concentration of 5 µg ml⁻¹. L-G broth, L-G agar (for the propagation of P1kc), nutrient broth and soft agar were prepared as described by Lambden & Drabble (1973).

Chemicals. Adenosine 5'-triphosphate (disodium salt, trihydrated) and β-NAD⁺ were obtained from Boehringer. Adenine sulphate, glutathione (reduced), guanine hydrochloride and Tris were obtained from Sigma. 6-Chloropurine ribotide (Cl-IMP) was from Calbiochem. Freund's complete adjuvant was from Difco. Ethyl methanesulphonate (EMS) was obtained from Eastman Kodak (Rochester, N.Y., U.S.A.), and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) from Ralph N. Emanuel (Wembley, HA0 1PY). ICR191E (2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamine]acridine dihydrochloride) was a gift from Dr H. J. Creech, The Institute for Cancer Research, Philadelphia, Pa, U.S.A. All other chemicals were from BDH.

Isolation of NG-induced mutants. Exponential phase cultures in L-G broth were centrifuged and the cells were resuspended to the same cell density in 0.1 M-Tris/maleate buffer, pH 6.0. NG was added (20 µg ml⁻¹ final concentration) for 30 min at 37 °C, then the bacteria were washed in sterile saline (0.85%, w/v, NaCl) and diluted 50-fold into minimal medium containing guanine (20 µg ml⁻¹). This medium allowed growth of the remaining prototrophs and phenotypic expression of guanine auxotrophs. After overnight incubation at 37 °C, the bacteria were washed twice with sterile saline and diluted into minimal medium containing adenine and thiamin to give an absorbance at 610 nm of about 0.03. This medium allowed growth of prototrophs and *pur* mutants, but not *gua* mutants. After a fourfold increase in cell mass (monitored by the increase in absorbance at 610 nm) to allow depletion of guanine pools, benzylpenicillin was added (final concentration 300 units ml⁻¹). When lysis was complete (no further decrease in absorbance), the bacteria were centrifuged, resuspended in sterile water, and serial dilutions were made to 10⁻² for replica-plating (Lambden & Drabble, 1973).

GuaB guaA double mutants were isolated from *guaB* mutants after NG mutagenesis. Penicillin enrichment of *guaB guaA* double mutants was made in minimal medium supplemented with xanthine. The double mutants were detected by replica-plating from guanine-supplemented to xanthine-supplemented minimal agar.

For all selections only one mutant of a particular phenotype was retained from each mutagen-treated culture.

Isolation of EMS-induced mutants. The method was described by Lambden & Drabble (1973).

Mutagen-induced reversion. The methods were described by Lambden & Drabble (1973), except for reversion induced by 2-aminopurine (2AP): 50 µl of a solution containing 1 mg 2AP ml⁻¹ was added to a sterile filter paper disc (13 mm diameter) placed at the centre of the agar plate.

Suppression of amber and ochre mutations. Inocula from overnight cultures of the *guaB* mutant to be tested and *Salmonella typhimurium* SL4528 [(*colEI*) *leu malB cysI gal his* (amber) (F'-1-*gal*⁺ *sup*-812)] (MacPhee & Stocker, 1969) were mixed in fresh L-G broth and incubated at 37 °C overnight. The cells were then washed twice with sterile saline and plated on to unsupplemented minimal agar. The plates were incubated for up to 4 d at 37 °C before colonies were scored. As a control, the *guaB* mutant was mixed with *S. typhimurium* SL4257 [(*colEI*) *leu malB cysI gal his* (amber) (F'-1-*gal*⁺)] using the same procedure; no growth was observed on unsupplemented minimal medium.

Enzyme assays. The growth of cultures, preparation of cell-free extracts and the assay for GMP synthetase (EC 6.3.4.1) were described by Lambden & Drabble (1973). IMP dehydrogenase (EC 1.2.1.14) was assayed according to Gilbert *et al.* (1979).

Complementation tests. Cell-free extracts were prepared as described by Lambden & Drabble (1973). Extracts (2 ml) were denatured by adding 2-mercaptoethanol to a final concentration of 0.1 M followed by 1.14 g solid guanidine hydrochloride over a 5 min period. The extracts were then left at 4 °C for 30 min. Denatured extracts from two different *guaB* mutants were mixed and then dialysed twice over a 16 h period against 100 vol. 0.02 M-sodium phosphate buffer, pH 7.4, containing 0.01 M-2-mercaptoethanol. After removing precipitated protein by centrifugation, the supernatant was assayed for IMP dehydrogenase activity.

Covalent modification of IMP dehydrogenase from *guaB* mutants. Cell-free extracts (1 ml, containing 5 mM

Table 1. Growth patterns of *pur* and *gua* mutants

Genotype	Growth* on minimal medium supplemented with				
	Unsupple- mented	Hypoxanthine + thiamin	Adenine + thiamin	Guanine	Xanthine
wild-type	+	+	+	+	+
<i>guaB</i> †	—	—	—	+	+
<i>guaA</i> ‡	—	—	—	+	—
<i>guaB guaA</i> §	—	—	—	+	—
<i>pur</i> (blocked before IMP)	—	+	+	+	+
<i>pur</i> (adenine branch)	—	—	+	—	—
*+, Growth; —, no growth.					
† HG1001 <i>guaB1001</i>	HG1019 <i>guaB1019</i>	HG1041 <i>guaB1041</i>	PL1096 <i>guaB76</i>		
HG1005 <i>guaB1005</i>	HG1024 <i>guaB1024</i>	HG1044 <i>guaB1044</i>	PL1105 <i>guaB85</i>		
HG1006 <i>guaB1006</i>	HG1025 <i>guaB1025</i>	PL1047 <i>guaB29</i>	PL1106 <i>guaB86</i>		
HG1010 <i>guaB1010</i>	HG1026 <i>guaB1026</i>	PL1063 <i>guaB43</i>	PL1138 <i>guaB105</i>		
HG1011 <i>guaB1011</i>	HG1027 <i>guaB1027</i>	PL1066 <i>guaB46</i>	MW1027 <i>guaB727</i>		
HG1015 <i>guaB1015</i>	HG1028 <i>guaB1028</i>	PL1072 <i>guaB52</i>	MW1038 <i>guaB738</i>		
HG1016 <i>guaB1016</i>	HG1029 <i>guaB1029</i>	PL1081 <i>guaB61</i>	MW1087 <i>guaB792</i>		
HG1017 <i>guaB1017</i>	HG1030 <i>guaB1030</i>	PL1087 <i>guaB67</i>	MW1102 <i>guaB807</i>		
HG1018 <i>guaB1018</i>	HG1040 <i>guaB1040</i>				
‡ HG1002 <i>guaA1002</i>	HG1014 <i>guaA1014</i>	HG1022 <i>guaA1022</i>	HG1047 <i>guaA1047</i>		
HG1009 <i>guaA1009</i>	HG1020 <i>guaA1020</i>	HG1043 <i>guaA1043</i>	PL1068 <i>guaA48</i>		
HG1012 <i>guaA1012</i>	HG1021 <i>guaA1021</i>				
§ HG1048 <i>guaB1029 guaA1048</i>	HG1054 <i>guaB727 guaA1054</i>	PL1141 <i>guaB43 guaA108</i>			
HG1049 <i>guaB1005 guaA1049</i>	HG1055 <i>guaB1001 guaA1055</i>	PL1143 <i>guaB46 guaA110</i>			
HG1050 <i>guaB1017 guaA1050</i>	HG1056 <i>guaB1016 guaA1056</i>	PL1145 <i>guaB67 guaA112</i>			
HG1051 <i>guaB1025 guaA1051</i>	HG1058 <i>guaB792 guaA1058</i>	PL1148 <i>guaB86 guaA114</i>			
HG1052 <i>guaB1018 guaA1052</i>	PL1140 <i>guaB29 guaA107</i>	PL1153 <i>guaB105 guaA121</i>			
HG1053 <i>guaB1010 guaA1053</i>					

reduced glutathione) of *guaB* mutants and the *guaA* mutant PL1068 were treated with 100 nmol chloropurine ribotide (Cl-IMP) and the resultant loss of IMP dehydrogenase activity was followed for the *guaA* mutant. When total inactivation of the native enzyme had occurred (approximately 2 min) all the extracts were left for a further 30 min to ensure complete reaction of the mutant enzymes with Cl-IMP. IMP was then added to a concentration of 2 mM. The preparations were dialysed three times for 8 h against 100 vol. 50 mM-potassium phosphate buffer, pH 7.4, containing 1 mM reduced glutathione and 2 mM-IMP. Mutant extracts (treated with Cl-IMP and untreated) were then denatured with guanidine hydrochloride (5 mM reduced glutathione replacing 2-mercaptoethanol) and mixed such that for each pair of mutants four types of hybrid enzyme were produced – covalently modified subunits from one or other of the mutants, all subunits modified or no subunit modified. IMP was present throughout to protect the hybrid enzymes from any Cl-IMP which had not been removed by prior dialysis. After renaturation, IMP dehydrogenase activity was determined for the various hybrid enzymes produced. Similar treatment was given to enzymes purified from HG1005 and PL1138 (purification described by Gilbert *et al.*, 1979). Covalent modification was achieved by adding 8 nmol portions of Cl-IMP until the reaction was complete (indicated by the absorbance at 290 nm reaching a maximum value). The enzymes were dialysed, denatured, mixed and renatured as described above, and finally assayed for IMP dehydrogenase activity.

Immunological methods. These were described by Gilbert *et al.* (1979).

RESULTS

Characterization of mutants

Preliminary classification of *gua* mutants (Table 1) was based on their growth on media supplemented with various purines. *GuaA* mutants have a specific requirement for guanine, but *guaB* mutants respond to guanine and xanthine. *GuaB guaA* double mutants have the same growth characteristics as *guaA* mutants.

The activities of IMP dehydrogenase and GMP synthetase were determined to confirm

Table 2. *Characteristics of guaB mutants**

Strain	Allele	Mutagen	Spontaneous reversion†	Mutagen-induced reversion†				Inferred class of mutation‡	Relative specific activities§			CRM production
				NG	EMS	2AP	ICR		IMP dehydrogenase	GMP synthetase		
HG1001	<i>guaB1001</i>	NG	+	+	++	—	—	BS	0	16	+	+
HG1005	<i>guaB1005</i>	NG	+	+	+++	—	—	BS	0	17	+	+
HG1006	<i>guaB1006</i>	NG	+	++	+++	++	—	BS	0	20	—	—
HG1010	<i>guaB1010</i>	NG	+	+	+++	—	—	BS	0	14	+	+
HG1011	<i>guaB1011</i>	NG	—	+	+++	—	—	BS	<0.1	10	—	—
HG1015	<i>guaB1015</i>	NG	+	+	+++	—	—	BS	0	12	—	—
HG1016	<i>guaB1016</i>	NG	+	+	+++	—	—	BS	0	13	+	+
HG1017	<i>guaB1017</i>	NG	++	+	+++	—	—	BS	0	14	+	+
HG1018	<i>guaB1018</i>	NG	+	+	+++	++	—	BS	0	21	+	+
HG1019	<i>guaB1019</i>	NG	—	+	+	—	—	BS	<0.1	19	+	+
HG1024	<i>guaB1024</i>	NG	+	+	+++	—	—	BS	0	18	—	—
HG1025	<i>guaB1025</i>	NG	+	+	+++	—	—	BS	0	18	+	+
HG1026	<i>guaB1026</i>	NG	+	+	+++	—	—	BS	0	12	—	—
HG1027	<i>guaB1027</i>	NG	—	+	+++	—	++	BS or FS	0	22	—	—
HG1028	<i>guaB1028</i>	NG	+	+	+++	—	—	BS	0	21	—	—
HG1029	<i>guaB1029</i>	NG	+	+	+++	—	—	BS	0	20	+	+
HG1030	<i>guaB1030</i>	NG	++	+	+++	—	—	BS	0	17	—	—
HG1040	<i>guaB1040</i>	EMS	—	+	++	—	—	BS	0	1	—	—
HG1041	<i>guaB1041</i>	EMS	+	+	+	++	—	BS	0	19	—	—
HG1044	<i>guaB1044</i>	EMS	++	+	+++	++	—	BS	<0.1	16	—	—
MW1027	<i>guaB727</i>	NG	+	+	+	—	—	BS	0	10	+	+
MW1038	<i>guaB738</i>	NG	++	+	+	++	—	BS	0	16	+	+
MW1087	<i>guaB792</i>	NG	+	+	++	—	—	BS	0	15	+	+
MW1102	<i>guaB807</i>	NG	+	+	+++	—	—	BS	0	18	+	+

* Characteristics of strains designated PL in Table 1 were given by Lambden & Drabble (1973).

† Mutagen-induced reversion is recorded as an increase over and above the number of spontaneous revertants for each strain. Symbols: —, no reversion; +, less than 20 colonies per plate; ++, 20 to 100 colonies; +++, more than 100 colonies.

‡ BS, Base substitution; FS, frameshift.

§ Specific activities relative to those for strain W3110 [IMP dehydrogenase: 8×10^{-3} $\mu\text{mol XMP min}^{-1}$ (mg protein) $^{-1}$; GMP synthetase: 12×10^{-3} $\mu\text{mol GMP min}^{-1}$ (mg protein) $^{-1}$].

|| Production of protein (CRM) cross reacting with anti-(IMP dehydrogenase) antibodies as determined by gel diffusion: +, CRM produced; —, no CRM.

Table 3. *Suppression of guaB mutations*

The procedure used is described in Methods.

<i>guaB</i> strains tested	Response
PL1063 (<i>guaB43</i>)*	Growth after 2 d
PL1081 (<i>guaB61</i>)*	Growth after 3–4 d
PL1047 (<i>guaB29</i>)*	No growth after 4 d
HG1001, HG1005, HG1006, HG1010, HG1011, HG1015, HG1016, HG1017, HG1018, HG1019, HG1024, HG1025, HG1026, HG1027, HG1028, HG1029, HG1030, HG1040, HG1041, HG1044, MW1038, MW1087, MW1102	No growth after 4 d

* These strains carry base substitution polar mutations (Lambden & Drabble, 1973).

the preliminary classification of *gua* mutants (Table 2). All *guaB* mutants were shown to lack a functional IMP dehydrogenase and produced, except for one mutant, derepressed levels of GMP synthetase. The exceptional mutant (HG1040) probably carries a polar mutation in *guaB* which exerts an effect on the adjacent *guaA* gene leading to low GMP synthetase activity. Polar *guaB* mutants of this type have been described previously by Lambden & Drabble (1973). Strains with a growth response characteristic of *guaA* mutations produced derepressed levels of IMP dehydrogenase but in no case was GMP synthetase detected (results not shown). No *guaB guaA* double mutant showed IMP dehydrogenase or GMP synthetase activity when grown under conditions for derepression.

The presence of protein (CRM) cross-reacting with anti-(IMP dehydrogenase) antibodies was detected by double immuno-gel diffusion (Table 2). The use of two monospecific antisera to IMP dehydrogenase, prepared by different methods (Gilbert *et al.*, 1979), gave identical results. Of the 24 mutants tested, 13 were CRM⁺. However, HG1001 and HG1017 gave fainter precipitin lines than other CRM⁺ strains. The mutant enzyme in these strains may be particularly susceptible to proteolytic degradation so that relatively low concentrations of the protein would be present in cell-free extracts. Spurs were observed between extracts of HG1029 and PL1068 (*guaA48*) suggesting that not all the antigenic sites of native IMP dehydrogenase are present on the protein produced by HG1029. All other cell-free extracts from *guaB* mutants produced complete fusion of precipitin lines with extracts containing native IMP dehydrogenase indicating antigenic identity of the mutant and native enzymes.

GuaB mutants were further classified according to their mutagen-induced reversion patterns (Table 2). The mutations appear to be of the base substitution type with the possible exception of *guaB1027* which may be a frameshift mutation. 2-Aminopurine causes transitions between AT and GC base pairs. Therefore the mutations which are reverted by 2-aminopurine have AT replacing the original GC as both NG and EMS cause a GC to AT exchange.

Suppression of *guaB* mutations was determined by conjugational transfer of an F' factor carrying an ochre suppressor gene (MacPhee & Stocker, 1969) from *S. typhimurium* to a series of *guaB* mutant strains (Table 3). Three strains with known base-substitution polar mutations [PL1047 (*guaB29*), PL1063 (*guaB43*) and PL1081 (*guaB61*)] isolated by Lambden & Drabble (1973) were included in this test to act as controls. Of these three polar mutations, *guaB43* and *guaB61* were suppressed while *guaB29* was not. PL1081 (*guaB61*) grew rather slowly. This suggests that *guaB43* is an ochre mutation whereas *guaB61* is an amber mutation because ochre suppressors are more effective against ochre than against amber mutations. *GuaB29* presumably generates a UGA termination codon as the ochre suppressor has no effect on this type of mutation. No other *guaB* strain tested responded to the suppressor suggesting that none carries amber or ochre mutations. The polar mutant HG1040 presumably has a UGA mutation.

Table 4. *Data from reciprocal three-factor crosses*

Reciprocal crosses were performed as described by Lambden & Drabble (1973). Wild-type recombinants (*guaB⁺guaA⁺*) were selected on unsupplemented minimal medium; *guaA⁺* recombinants (*guaB⁺guaA⁺* and *guaB guaA⁺*) were selected on xanthine-supplemented medium.

Donor allele	Recipient alleles		Transductants selected ($10^6 \times$ f.o.t.)*		B^+A^+	Inferred order
			<i>guaB⁺guaA⁺</i>	<i>guaA⁺</i>	A ⁺ (%)	
<i>guaB1001</i>	<i>guaB1001</i>	<i>guaA1055</i>	0.0000	2.40	0.00	
<i>guaB1005</i>	<i>guaB1005</i>	<i>guaA1049</i>	0.0000	3.40	0.00	
<i>guaB1016</i>	<i>guaB1016</i>	<i>guaA1056</i>	0.0000	1.30	0.00	
<i>guaB1017</i>	<i>guaB1017</i>	<i>guaA1050</i>	0.0000	5.20	0.00	
<i>guaB1018</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0000	4.60	0.00	
<i>guaB792</i>	<i>guaB792</i>	<i>guaA1058</i>	0.0000	6.70	0.00	
<i>guaB29</i>	<i>guaB29</i>	<i>guaA107</i>	0.0000	0.80	0.00	
<i>guaB43</i>	<i>guaB43</i>	<i>guaA108</i>	0.0000	0.87	0.00	
<i>guaB67</i>	<i>guaB67</i>	<i>guaA112</i>	0.0000	1.20	0.00	
<i>guaB86</i>	<i>guaB86</i>	<i>guaA114</i>	0.0000	5.20	0.00	
<i>guaB105</i>	<i>guaB105</i>	<i>guaA121</i>	0.0000	2.50	0.00	
<i>guaB46</i>	<i>guaB46</i>	<i>guaA110</i>	0.0000	3.20	0.00	
<i>guaB1001</i>	<i>guaB105</i>	<i>guaA121</i>	0.0140	0.42	3.33	
<i>guaB105</i>	<i>guaB1001</i>	<i>guaA1055</i>	0.0070	0.82	0.85	<i>B1001-B105-guaA</i>
<i>guaB1005</i>	<i>guaB105</i>	<i>guaA121</i>	0.0610	0.68	8.97	
<i>guaB105</i>	<i>guaB1005</i>	<i>guaA1049</i>	0.0008	0.48	0.17	<i>B1005-B105-guaA</i>
<i>guaB1016</i>	<i>guaB105</i>	<i>guaA121</i>	0.0060	0.12	5.00	
<i>guaB105</i>	<i>guaB1016</i>	<i>guaA1056</i>	0.0088	2.40	0.37	<i>B1016-B105-guaA</i>
<i>guaB1018</i>	<i>guaB105</i>	<i>guaA121</i>	0.0800	3.50	2.29	
<i>guaB105</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0008	0.31	0.26	<i>B1018-B105-guaA</i>
<i>guaB792</i>	<i>guaB105</i>	<i>guaA121</i>	0.0800	7.07	1.13	
<i>guaB105</i>	<i>guaB792</i>	<i>guaA1058</i>	0.0065	0.93	0.70	<i>B792-B105-guaA</i>
<i>guaB29</i>	<i>guaB105</i>	<i>guaA121</i>	0.0091	0.89	1.02	
<i>guaB105</i>	<i>guaB29</i>	<i>guaA107</i>	0.0061	2.13	0.29	<i>B29-B105-guaA</i>
<i>guaB46</i>	<i>guaB105</i>	<i>guaA121</i>	0.0550	3.31	1.66	
<i>guaB105</i>	<i>guaB46</i>	<i>guaA110</i>	0.0400	11.12	0.36	<i>B46-B105-guaA</i>
<i>guaB67</i>	<i>guaB105</i>	<i>guaA121</i>	0.0400	0.60	6.67	
<i>guaB105</i>	<i>guaB67</i>	<i>guaA112</i>	0.0234	2.09	1.12	<i>B67-B105-guaA</i>
<i>guaB86</i>	<i>guaB105</i>	<i>guaA121</i>	0.0102	1.76	0.58	
<i>guaB105</i>	<i>guaB86</i>	<i>guaA114</i>	0.1320	9.76	1.36	<i>B105-B86-guaA</i>
<i>guaB1018</i>	<i>guaB46</i>	<i>guaA110</i>	0.0448	5.60	0.80	
<i>guaB46</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0030	2.30	0.13	<i>B1018-B46-guaA</i>
<i>guaB1018</i>	<i>guaB1005</i>	<i>guaA1049</i>	0.0004	4.24	0.01	
<i>guaB1005</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0130	0.30	4.33	<i>B1005-B1018-guaA</i>
<i>guaB1018</i>	<i>guaB1017</i>	<i>guaA1050</i>	0.0750	10.00	0.75	
<i>guaB1017</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0030	0.48	0.63	<i>B1018-B1017-guaA</i>
<i>guaB1018</i>	<i>guaB43</i>	<i>guaA108</i>	0.0006	2.00	0.03	
<i>guaB43</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0040	0.26	1.54	<i>B43-B1018-guaA</i>
<i>guaB1018</i>	<i>guaB29</i>	<i>guaA107</i>	0.0827	5.30	1.56	
<i>guaB29</i>	<i>guaB1018</i>	<i>guaA1052</i>	0.0050	0.70	0.71	<i>B1018-B29-guaA</i>
<i>guaB1017</i>	<i>guaB43</i>	<i>guaA108</i>	0.0047	4.90	0.10	
<i>guaB43</i>	<i>guaB1017</i>	<i>guaA1050</i>	0.0041	0.32	1.28	<i>B43-B1017-guaA</i>
<i>guaB1017</i>	<i>guaB67</i>	<i>guaA112</i>	0.0052	3.50	0.15	
<i>guaB67</i>	<i>guaB1017</i>	<i>guaA1050</i>	0.0064	0.13	4.92	<i>B67-B1017-guaA</i>
<i>guaB1017</i>	<i>guaB1001</i>	<i>guaA1055</i>	0.0020	0.23	0.87	
<i>guaB1001</i>	<i>guaB1017</i>	<i>guaA1050</i>	0.0026	0.10	2.60	<i>B1001-B1017-guaA</i>
<i>guaB1016</i>	<i>guaB67</i>	<i>guaA112</i>	0.0622	6.00	1.04	
<i>guaB67</i>	<i>guaB1016</i>	<i>guaA1056</i>	0.0100	0.57	1.75	<i>B67-B1016-guaA</i>
<i>guaB1005</i>	<i>guaB67</i>	<i>guaA112</i>	0.0340	1.60	2.13	
<i>guaB67</i>	<i>guaB1005</i>	<i>guaA1049</i>	0.0100	1.00	1.00	<i>B1005-B67-guaA</i>

Table 4. (cont.)

Donor allele	Recipient alleles		Transductants selected ($10^6 \times$ f.o.t.)*		B^+A^+	Inferred order
			<i>guaB⁺guaA⁺</i>	<i>guaA⁺</i>	A^+ (%)	
<i>guaB1001</i>	<i>guaB792</i>	<i>guaA107</i>	0.0150	0.70	2.14	<i>B1001-B792-guaA</i>
<i>guaB792</i>	<i>guaB1001</i>	<i>guaA1055</i>	0.0150	1.60	0.94	
<i>guaB792</i>	<i>guaB86</i>	<i>guaA114</i>	0.0120	0.62	1.93	<i>B792-B86-guaA</i>
<i>guaB86</i>	<i>guaB792</i>	<i>guaA1058</i>	0.0500	7.20	0.69	
<i>guaB46</i>	<i>guaB86</i>	<i>guaA114</i>	0.0030	0.11	2.72	<i>B46-B86-guaA</i>
<i>guaB86</i>	<i>guaB46</i>	<i>guaA110</i>	0.0020	1.20	0.17	
<i>guaB46</i>	<i>guaB29</i>	<i>guaA107</i>	0.0016	0.12	1.33	<i>B46-B29-guaA</i>
<i>guaB29</i>	<i>guaB46</i>	<i>guaA110</i>	0.0014	0.23	0.61	
<i>guaB1005</i>	<i>guaB46</i>	<i>guaA110</i>	0.0840	1.62	5.18	<i>B1005-B46-guaA</i>
<i>guaB46</i>	<i>guaB1005</i>	<i>guaA1049</i>	0.0010	1.00	0.10	
<i>guaB1017</i>	<i>guaB105</i>	<i>guaA121</i>	0.0748	4.09	1.82	<i>B1017-B105-guaA</i>
<i>guaB105</i>	<i>guaB1017</i>	<i>guaA1050</i>	0.0270	2.37	1.14	

* f.o.t. (frequency of transduction) is the number of transductants obtained per phage added.

Mapping of *guaB* mutations by three-factor crosses

Mutations were mapped at the *guaB* locus by transductional three-factor crosses using *guaA* as the outside reference marker. The principle of the cross was described by Lambden & Drabble (1973). The recipient strain in the crosses carried an additional mutation in the closely linked *guaA* gene. Pairs of *guaB* mutations were thus ordered with respect to the third reference *guaA* mutation. Wild-type recombinants (*guaB⁺guaA⁺*) were selected on unsupplemented minimal medium whereas *guaA⁺* recombinants (*guaB⁺guaA⁺* and *guaB⁻guaA⁺*) were selected on xanthine-supplemented medium. Wild-type recombinants were expressed as a percentage of the total *guaA⁺* recombinants (Table 4).

The order of *guaB* mutations with respect to the *guaA* marker can be deduced from the recombination frequencies for a particular pair of reciprocal crosses. The higher value of B^+A^+/A^+ was taken as an indication of the distance between *guaB* mutations (Fig. 1). The map (Fig. 1) also incorporates data of Lambden & Drabble (1973). A major feature of the map is a clustering of mutations in the operator distal region delineated by *guaB1001* and *guaB105*. This may indicate an important role for the amino acid sequence coded by this region in the correct formation of enzyme tertiary structure. There is no consistent pattern of mutations giving rise to CRM.

Complementation in vitro between *guaB* mutants

No IMP dehydrogenase activity was observed when cell-free extracts of *guaB* mutants were individually denatured with guanidine hydrochloride and renatured, nor when they were mixed in the absence of denaturing conditions. Mixing the extract of any CRM⁺ mutant with that of PL1138 (*guaB105*) in denaturing conditions resulted in restoration of enzyme activity after renaturation (Table 5). The activity observed depended on the particular CRM⁺ mutant used to complement PL1138. The combination of extracts from PL1138 and HG1005 produced the most active enzyme. Extracts of CRM⁻ mutants, when mixed with an extract of PL1138, produced no enzyme activity. No other combination of extracts resulted in complementation.

Heat stability of native and hybrid forms of IMP dehydrogenase

The heat stability of native enzyme, reconstituted native enzyme and two hybrid enzymes (PL1138-HG1001 and PL1138-HG1005 extract combinations) was examined (Fig. 2). Under the conditions used both native and reconstituted IMP dehydrogenases were stable

Table 5. Complementation *in vitro* between *guaB* mutants

Complementation was performed as described in Methods. The results show the specific activities of IMP dehydrogenase (%) compared with denatured and renatured native enzyme (a cell-free extract of derepressed PL1068) which has a specific activity of $0.3 \mu\text{mol XMP min}^{-1} (\text{mg protein})^{-1}$ (=100%).

		CRM ⁺														CRM ⁻																
		PL1138	PL1072	PL1096	PL1105	HG1001	HG1005	HG1010	HG1016	HG1017	HG1018	HG1019	HG1025	HG1029	MW1027	MW1038	MW1087	MW1102	HG1006	HG1011	HG1015	HG1024	HG1026	HG1027	HG1028	HG1030	HG1040	HG1041	HG1044			
CRM ⁺	PL1138	0																														
	PL1072	27	0																													
	PL1096	23	0	0																												
	PL1105	30	0	0	0																											
	HG1001	9	0	0	0	0																										
	HG1005	35	0	0	0	0	0																									
	HG1010	17	0	0	0	0	0	0																								
	HG1016	31	0	0	0	0	0	0	0																							
	HG1017	13	0	0	0	0	0	0	0	0																						
	HG1018	27	0	0	0	0	0	0	0	0	0																					
	HG1019	15	0	0	0	0	0	0	0	0	0	0																				
	HG1025	33	0	0	0	0	0	0	0	0	0	0	0																			
	HG1029	28	0	0	0	0	0	0	0	0	0	0	0	0																		
	MW1027	21	0	0	0	0	0	0	0	0	0	0	0	0	0																	
	MW1038	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0																
MW1087	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0																
MW1102	19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0															
CRM ⁻	HG1006	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0													
	HG1011	0	-	-	-	-	-	0	-	-	-	0	0	-	-	0	-	-	-	0		0										
	HG1015	0	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	0	-	0	0										
	HG1024	0	-	-	-	-	-	-	0	-	-	-	-	-	-	0	-	-	-	-	-	-	0									
	HG1026	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0								
	HG1027	0	-	-	-	0	0	-	0	-	-	0	0	-	-	-	-	-	-	-	-	0	0	-	0							
	HG1028	0	-	-	-	0	0	-	0	-	-	0	0	-	-	0	-	-	-	0	0	-	-	-	-	0						
	HG1030	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0					
	HG1040	0	-	-	-	0	-	-	0	-	-	0	-	-	-	0	-	-	-	0	-	0	-	-	-	-	-	0				
	HG1041	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	0	0			
	HG1044	0	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	0	-	-	-	-	-	-	-	-	0	0	0		

-, Not tested.

for up to 30 min at 70 °C and both had the same susceptibility to heat inactivation at higher temperatures (Fig. 2). This suggests that the tertiary structure of the enzyme is unchanged by denaturation and renaturation. The hybrid enzymes were more heat labile. The more enzymically active hybrid (PL1138–HG1005) showed the greater heat stability. Loss of enzyme activity at each temperature was first order with respect to time (Fig. 2*b*).

Kinetic properties of native and hybrid IMP dehydrogenases

Native and hybrid (PL1138–HG1001 and PL1138–HG1005) IMP dehydrogenases were purified as described by Gilbert *et al.* (1979). The kinetic constants (Table 6) of these enzymes were determined at a fixed concentration of NAD for the K_m^{IMP} and vice versa for K_m^{NAD} . The K_i^{GMP} was determined at 0.1 and 0.2 mM-GMP. The kinetic constants for native, reconstituted native and the hybrid enzymes were similar; therefore the low specific activities of the hybrid enzymes (Table 5) are not related to an unusually high K_m for one or both of the substrates.

Fig. 2. Heat stability of native and hybrid forms of IMP dehydrogenase. Cell-free extracts were prepared, denatured and renatured as described in Methods. Samples (2 ml, 5 mg protein ml⁻¹) were incubated either for 10 min at various temperatures (a) or for various times at 78 °C (b). Portions (0.3 ml) were withdrawn at the end of the incubation, cooled to 0 °C and assayed for IMP dehydrogenase activity. ○, Native enzyme present in a cell-free extract from PL1068 (*guaA48*); ●, reconstituted native enzyme from PL1068 (*guaA48*); □, hybrid enzyme from mixed extracts of PL1138 and HG1005; ■, hybrid enzyme from mixed extracts of PL1138 and HG1001.

Table 6. Kinetic constants for native and hybrid IMP dehydrogenases

The reaction mixtures contained, in 1 ml: Tris/HCl (pH 7.85), 50 μmol; KCl, 33 μmol; reduced glutathione, 5 μmol; and (for the determination of K_m^{IMP}) IMP, 0.01 to 0.20 μmol, and NAD, 0.20, 0.40 or 1.25 μmol, or (for the determination of K_m^{NAD}) NAD, 0.1 to 2.0 μmol, and IMP, 0.02, 0.10 or 0.20 μmol. K_i^{GMP} was determined using 0.10 and 0.20 μmol GMP. The results show the kinetic constants ± the standard error of the mean, with the number of determinations in parentheses.

Enzyme	Kinetic constants (μM)		
	K_m^{IMP}	K_m^{NAD}	K_i^{GMP}
Native	11.1 ± 0.6 (6)	330 ± 10 (4)	56 ± 3 (3)
Denatured and renatured native	11.3 ± 0.12 (3)	341 ± 26 (3)	63 ± 16 (3)
HG1005-PL1138 hybrid	10.1 ± 0.12 (3)	252 ± 21 (3)	56 ± 8 (3)
HG1001-PL1138 hybrid	7.5 ± 0.15 (3)	202 ± 23 (3)	60 ± 11 (3)

Covalent modification of hybrid IMP dehydrogenase

Native IMP dehydrogenase and the enzyme from several of the CRM⁺ *guaB* mutants can be covalently modified at the active site by Cl-IMP (Gilbert & Drabble, 1978). Thus, the absorption spectrum of Cl-IMP undergoes a characteristic change on incubation of the reagent with purified native or mutant enzymes and the native enzyme becomes inactive. Specific modification at the IMP binding site of the enzyme is suggested by the considerable retardation of this reaction by IMP, and by the non-reactivity of chloro-inosine. Amongst the mutant enzymes modified by Cl-IMP were those produced by HG1005, HG1019, PL1072, PL1105 and PL1138.

Enzyme activity was determined for hybrid enzymes containing subunits which had been covalently modified with Cl-IMP (Table 7). Enzyme activity was absent when either all subunits had been modified or only those subunits derived from PL1138. When subunits from PL1138 had not been modified enzyme activity (75 to 90% of that of the untreated hybrid enzyme) was observed. These results suggest that in hybrid enzymes the subunits derived from PL1138 are responsible for the catalytic reaction. The complementary subunit may be holding subunits from PL1138 in the correct conformation for catalysis. The reduced activity of the hybrid enzymes with modification to subunits other than those of PL1138 may be a consequence of incomplete removal of Cl-IMP during dialysis.

Table 7. Covalent modification of hybrid IMP dehydrogenase by *Cl*-IMP

The experiments were performed as described in Methods. Purified enzyme (Gilbert *et al.*, 1979) was used in the first experiment listed; in all others, hybrid enzyme was formed from cell-free extracts. In each case, the enzyme activity of the modified enzyme is shown relative to that of the unmodified control preparation (= 100%).

Hybrid enzyme	Subunits modified	Enzyme activity (%)
HG1005 × PL1138	None	100
	PL1138	0
	HG1005	84
	HG1005 and PL1138	0
HG1005 × PL1138	None	100
	PL1138	0
	HG1005	78
	HG1005 and PL1138	0
PL1105 × PL1138	None	100
	PL1138	0
	PL1105	90
	PL1105 and PL1138	0
HG1019 × PL1138	None	100
	PL1138	0
	HG1019	75
	HG1019 and PL1138	0
PL1072 × PL1138	None	100
	PL1138	0
	PL1072	81
	PL1072 and PL1138	0

DISCUSSION

Complementation *in vitro* was observed only by denaturing and renaturing mixed extracts of a CRM⁺ *guaB* mutant and PL1138 (*guaB105*). The lack of complementation by CRM⁻ strains may arise because these mutants produce altered IMP dehydrogenase proteins which are susceptible to proteolysis and are therefore absent from cell-free extracts (Greeb *et al.*, 1971; Schlesinger, 1974), although in some strains (e.g. HG1040) the mutations may prevent transcription and/or translation of *guaB*. The requirement for denaturing conditions indicates that the mutant strains produce IMP dehydrogenase in an aggregated form. For hybridization, the proteins must first be dissociated so that free monomers from different molecules can come together.

Intrallelic complementation amongst *guaB* mutants of *S. typhimurium* has been demonstrated by Schafer *et al.* (1974). The complementation between *guaB* mutants of *E. coli* likewise appears to be interallelic which implies that the subunits of IMP dehydrogenase are identical. The activity of the hybrid enzyme depends on which *guaB* mutant is paired with PL1138. For intercistronic complementation the expected enzyme activities would be fairly constant, irrespective of mutant pairing, at about 50% of the control value. As the IMP dehydrogenase activity of the hybrids that we examined never approached 50% of the control, interallelic complementation is again signified. To explain these observations by intercistronic complementation would require the added assumption that the variable and low recovery of enzyme activity arises from different degrees of proteolysis of the enzyme monomers or from altered sensitivity of mutated enzymes to denaturing conditions. One mutation (*guaB105*) would correspond to one cistron whereas 16 mutations would be placed in the other. Two cistrons of approximately equal size [mol. wt of the two dissimilar subunits suggested by Powell (1973) are 60000 and 44000] would not be expected to differ so significantly in their susceptibility to mutation, but as *guaB105* maps to the right of all complementing mutations (Fig. 1) an intercistronic boundary between *guaB105* and *guaB85*

is conceivable. However, interallelic complementation has been observed between mutations in the operator distal portion of *lacZ* and mutations mapping elsewhere in the gene (Ullman *et al.*, 1967).

The hybrid enzyme produced by complementing PL1138 with HG1001 is more heat labile than that formed from PL1138 with HG1005, which, in turn, is more heat labile than native reconstituted enzyme. This strongly indicates interallelic complementation for IMP dehydrogenase rather than intercistronic complementation which would yield hybrid enzyme with the same heat stability as native enzyme. Interallelic complementation is also favoured by the findings that no CRM⁻ mutation mapping operator-proximal to *guaB85* complements *guaB105*, and *guaB86* (CRM⁻) does not complement any mutation operator-proximal to *guaB85*. However, the situation could again be confused by intracellular proteolysis of mutant forms of the enzyme prior to hybridization. Taken together with the finding of Krishnaiah (1975) and Gilbert *et al.* (1979) that the subunits of purified IMP dehydrogenase are identical, the evidence presented in this report is most compatible with a system of interallelic complementation.

Why does only one mutation (*guaB105*) complement all other CRM⁺ mutations? *GuaB105* was induced by EMS whereas the others were induced by NG. However, as both mutagens act in a similar manner, they are unlikely to produce mutations of different complementing types. Attempts to isolate other mutants similar to PL1138 (*guaB105*) have so far proved unsuccessful. This may indicate that the replacement of only a small number of specific amino acids confers the phenotype characteristic of *guaB105*. Perhaps significantly, strain PL1138 shows slightly leaky growth on guanine-free media; leaky mutants most often provide the best complementation (Fincham *et al.*, 1979).

Proteolytic enzymes cleave the monomer of IMP dehydrogenase into two polypeptides of unequal size (Gilbert *et al.*, 1979). This indicates the presence of a region in the protein particularly susceptible to proteolysis, possibly a 'bridge' between two globular domains. Strain PL1138 may produce a protein with an amino acid substitution in the smaller domain and the complementary mutants may produce proteins with amino acid substitutions in the larger domain. Alternatively, complementation may be similar to that observed for certain glutamate dehydrogenase mutants of *Neurospora crassa*. This allosteric enzyme has two conformational states, active and inactive. A number of mutant enzymes, all frozen in the inactive state, when mixed with a mutant with a defect in the active site were able to change conformation and regain activity (Brett *et al.*, 1976; Watson & Wootton, 1978). Similarly, repairable variant enzymes having conformationally deformed active sites have also been observed for tryptophan synthetase of *E. coli* (Kida & Crawford, 1974). By analogy with these examples, the unique *guaB* mutant PL1138 would produce enzyme frozen in an inactive conformation but capable of being repaired by other monomers defective at the active site. Covalent modification of the hybrid enzymes supports this conclusion by demonstrating that only the monomers from PL1138 are catalytically active in the hybrids. The 'mutual correction' theory of Crick & Orgel (1964) offers a less satisfactory explanation for the type of complementation described here.

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