Structural and Functional Roles of the Cysteine Residues in the α Subunit of the *Escherichia coli* Tryptophan Synthetase. II. Functional Roles of the Cysteine Residues*

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ABSTRACT: From the chemical and enzymatic properties of the *N*-ethylmaleimide derivatives of the α subunit described here and in the preceding report, certain correlations of the structural and functional roles of the three cysteine residues in this enzyme were possible. *N*-Ethylmaleimide labeling at any cysteine residue in the protein renders it completely inactive by itself. Cysteine-80-labeled protein, which is immunochemically similar to the normal enzyme, also apparently forms a tetrameric ($\alpha_2\beta_2$) structure with the β_2 subunit. This complex, however, when tested in any of its three reactions, appears to be only about 50% as active as the normal $\alpha_2\beta_2$ tetramer. Reaction of *N*-ethylmaleimide at cysteine-117 or cysteine-153 of the protein, which totally destroys any interaction with the antibody, also leads to an anomalous association with the β_2 subunit. A large, inactive aggregate is formed with an estimated molecular weight three- to fourfold larger than the normal $\alpha_2\beta_2$ structure. Interactions between unlabeled protein and some of the labeled protein derivatives, which probably occur during the labeling process, are also evident in activity assays without the β_2 subunit. The activity of the unlabeled enzyme, present in unfractionated, inactive preparations, is only apparent when the cysteine-117 or cysteine-153-labeled molecules are removed. No such interactions were seen in activity assays with the β_2 subunit. From the pattern of activity loss (with the β_2 subunit) as a function of *N*-ethylmaleimide binding, a model for the *N*-ethylmaleimide reaction with the α subunit is proposed. It is suggested that at low concentrations, *N*-ethylmaleimide reacts predominantly at cysteine-117 and/or cysteine-153 to yield one of the inactive singly labeled protein species and some doubly labeled inactive protein forms. At intermediate levels of *N*-ethylmaleimide, the partially active, cysteine-80-labeled derivative is formed. At saturating *N*-ethylmaleimide concentrations, the other singly and doubly labeled inactive derivatives are produced.

The previous paper (Malkinson and Hardman, 1969) described the fractionation and some of the chemical and physical properties of the *N*-ethylmaleimide derivatives of the α subunit of the *Escherichia coli* tryptophan synthetase. On the basis of these data, certain structural roles for the different cysteine residues were suggested. To correlate these roles with their functional roles, the enzymatic properties of these derivatives alone and in combination with the β_2 subunit have been examined and are presented here.

Methods and Materials

Enzyme Assays. In → Trp[1] activity was determined according to Smith and Yanofsky (1962). InGP → Trp activity was measured in two ways: in kinetic experiments, the spectrophotometric assay described by Creighton and Yanofsky (1966) was employed; alternatively, the loss of [^{14}C]indoleglycerol phosphate was determined after incubation in a reaction mixture containing L-serine, pyridoxal phosphate, and NaCl (Smith and Yanofsky, 1962). Residual [^{14}C]indoleglycerol phosphate was oxidized by periodate (Yanofsky, 1956), extracted with ethyl acetate, and counted. Measurement of InGP → In activity with the α subunit alone was performed with [^{14}C]indoleglycerol phosphate as described by Hardman and Yanofsky (1965). When this reaction was measured in the presence of the β_2 subunit, 0.06 M hydroxylamine hydrochloride was included.

Association of α and β_2 Subunits. The procedures for obtaining and measuring the physical association of the two subunits were developed by Creighton and Yanofsky (1966). To study association by sucrose gradient centrifugation, subunits were preincubated at either 25° for 30 min or 37° for 15 min in a mixture containing L-serine and 0.02 mM pyridoxal phosphate. No differences were observed when sucrose solutions were made in either 0.05 M Tris-HCl buffer (pH 7.8) or in water. A 0.1–0.25-ml reaction mixture, also containing 0.03 ml of catalase solution (Worthington) as a marker in all gradients, was layered on to 4.6 ml of a 5–20% sucrose gradient prepared according to Martin and Ames (1961). Centrifugation in a SW-50L rotor was done at 4–6° in a Spinco Model L-2 for 10–14 hr at 39,000 rpm. Six drop fractions were collected and analyzed for radioactivity and enzymatic activity.

Association of subunits by Sephadex gel filtration was performed on a Sephadex G-200 column (0.6 × 40 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.0), containing 0.15 mM L-serine and 0.02 mM pyridoxal phosphate. Protein samples, containing these same concentrations

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[1] See Malkinson and Hardman (1969) for list of abbreviations.

TABLE I: Enzymatic Activities of *N*-Ethylmaleimide-, *p*-Hydroxymercuribenzoate- and 5,5′-Dithiobis(-2-nitrobenzoic Acid) Labeled α Subunit.

Reagent	Moles of Reagent/Mole of Protein	Reaction	Rel Sp Act. (%)
N-Ethylmaleimide	1.01	InGP → In ($-\beta_2$ subunit)	0
	1.10	InGP → In	39
	1.10	In → Trp ($+\beta_2$ subunit)	36
	1.00	InGP → Trp	38
p-Hydroxymercuribenzoate	1.10	InGP → Trp ($+\beta_2$ subunit)	31
5,5′-Dithiobis(-2-nitrobenzoic acid)	0.91	In → Trp ($+\beta_2$ subunit)	37

of cofactors, were layered on to the column in a volume of 0.2 ml without any preincubation. Fractions of 0.2–0.5 ml were collected and analyzed for radioactivity and enzymatic activity.

Materials. Crude extracts of *E. coli* mutants A2 and A3 (kindly supplied by C. Yanofsky), which contain defective α subunits, were employed as the source of the β_2 subunit and the indoleglycerol phosphate synthetase, respectively. Indoleglycerol phosphate was prepared enzymatically from N^5′-phosphoribosyl anthranilate and 1-(*O*-carboxyphenylamino)-1-deoxyribulose 5-phosphate with indoleglycerol phosphate synthetase (Creighton and Yanofsky, 1969). [^{14}C]Indoleglycerol phosphate was prepared from [^{14}C]indole according to Hardman and Yanofsky (1965).

All other procedures have been described before (Malkinson and Hardman, 1969).

Experimental Results

Enzymatic Activities of N-Ethylmaleimide Derivatives of the α Subunit. *N*-Ethylmaleimide reaction with the α subunit produces a number of *N*-ethylmaleimide-labeled forms of the protein. Because some of these appeared to be similar in structure to the normal enzyme and others totally different, certain structural roles of the different cysteine residues were suggested (Malkinson and Hardman, 1969). To determine a relationship, if any, between the structural and functional roles of these cysteine residues, the enzymatic capacity of these α-subunit derivatives was examined (Table I). The specific activity of the native enzyme in each of the tryptophan synthetase reactions agreed well with the published values (Crawford and Yanofsky, 1959; Hatanaka *et al.*, 1962); these values were set at 100% for each of the reactions and the activities of treated protein are expressed as relative specific activities. It is seen (Table I) that although the α subunit, fully labeled with *N*-ethylmaleimide, contains no activity by itself, it still retains 32–39% of its specific activity when measured in the presence of the β_2 subunit. This is true regardless of the reaction measured. The average value for these activities was 38%. The activity loss upon complete labeling with *p*-hydroxymercuribenzoate or 5,5′-dithiobis(-2-nitrobenzoic acid) is similar to that found with *N*-ethylmaleimide; this correlates well with the reactivity of all three reagents (Malkinson and Hardman, 1969).

To further explore these results, fractionation of *N*-ethylmaleimide-treated protein by polyacrylamide disc electrophoresis (Malkinson and Hardman, 1969) was undertaken. The activity in the InGP → Trp reaction in the presence of the β_2 subunit is shown in Figure 1; the profiles for the In → Trp and InGP → In activities (with the β_2 subunit) are similar. The InGP → In activity of combined fractions of peak I and peak II, without the β_2 subunit, also present a similar pattern. Nearly all the activity is contained in peak I.

The results of activity assays in the absence of the β_2 subunit are shown in Table II and indicate that unfractionated

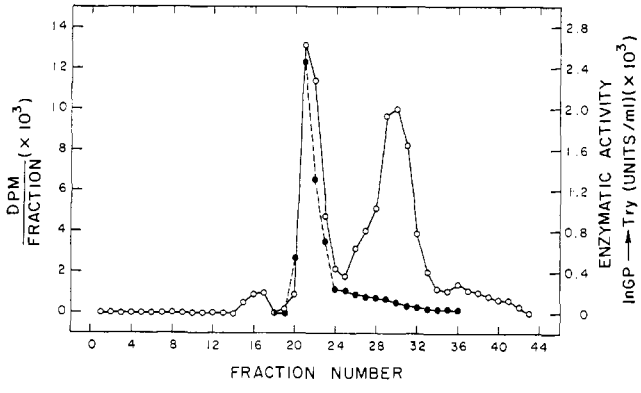

FIGURE 1: Activity (InGP → Trp, with β_2 subunit) in fractions obtained upon polyacrylamide electrophoresis of *N*-ethylmaleimide-treated α subunit (*N*-ethylmaleimide/protein 1.24); (○—○) disintegrations per minute per fraction; (●—●) activity.

TABLE II: Activity of *N*-Ethylmaleimide-Labeled α-Subunit Derivatives in Absence of β_2 Subunit.

	Rel Sp Act. (%)
Normal α subunit	100
Unfractionated *N*-ethylmaleimide subunit (*N*-ethylmaleimide/protein 1.22)	0
Peak I (*N*-ethylmaleimide/protein 0.64)	39
Peak II (*N*-ethylmaleimide/protein 1.56)	6

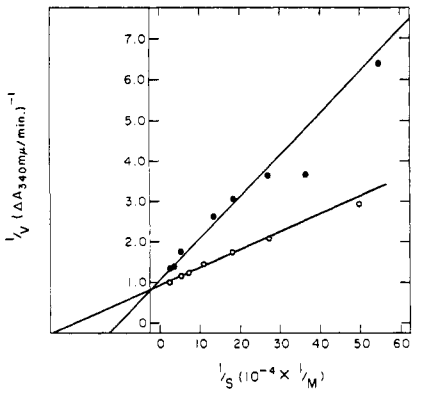

FIGURE 2: Lineweaver–Burk plot of the InGP → Trp activity of the native α subunit (○—○) and peak I (●—●).

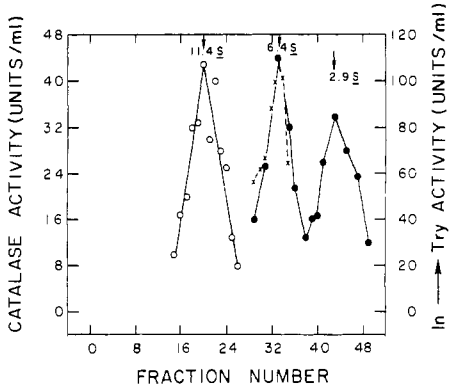

FIGURE 3: Sucrose density gradient of peak I in the presence of the β_2 subunit. A 0.1-ml aliquot of peak I (*N*-ethylmaleimide/protein 0.61) was layered onto a 5–20% sucrose gradient after prior incubation for 30 min at 25° with 200 units of β_2 subunit, pyridoxal phosphate, and L-serine. A 0.03-ml aliquot of 0.02 M potassium phosphate buffer (pH 7.0), containing 0.005 M EDTA and 0.25 mM dithiothreitol, was added to each fraction. Alternate fractions were assayed for α-subunit and β_2-subunit activity in the In → Trp reaction; the activity of each subunit was measured in the presence of the other subunit. (○—○) Catalase activity; (●—●) peak I activity; (X—X) β_2-subunit activity.

and totally inactive *N*-ethylmaleimide-treated protein yields active forms (peak I) when separated into the two fractions. Furthermore, the relative specific activity of peak I (39%) correlates well with the amount of unlabeled protein as judged by the *N*-ethylmaleimide/protein ratio of 0.61. This behavior is analogous to the response of unfractionated and peak I protein to *N*-ethylmaleimide labeling (Malkinson and Hardman, 1969) and suggests that the masking of unlabeled protein by peak II material exists not only during the labeling process but during the InGP → In assays as well.

Activity measurements in the presence of the β_2 subunit do not suggest such interactions, however. This is based primarily on the fact that there is little difference in total activity before and after fractionation. The recovery of this activity in peak I is 85–90% that of unfractionated protein. In other words, the unlabeled protein appears to function similarly with the β_2 subunit in both unfractionated and peak I states. The assignment of the relative specific activity to each form in peak I is difficult since they have not been resolved as yet. The specific activity in the individual fractions of peak I ranged from 60 to 100% that of the normal enzyme. The fractions with the highest specific activity (90–100%) were always at the leading edge, and those with lowered specific activity (60–75%) were nearer the trailing edge. The average specific activity of combined peak I fractions was routinely about 75%. The possibility that the lowered activity of some of the peak I fractions is due to 25–40% contamination with inactive peak II material does not seem likely. Although combined peak I fractions were often found to contain traces of peak II protein by analytical polyacrylamide disc electrophoresis, the level of this contamination was difficult to quantitate by this technique. Several fractionations, however, in which no contamination of peak I by peak II was detectable electrophoretically, produced this same pattern of specific activity. Thus, fully and partially active enzyme are present in peak I and result in an average specific activity of 75%. From these considerations and the fact that peak I contains equal amounts of unlabeled and labeled protein, the activity of the labeled protein would be expected to be about 50% of the normal activity with the β_2 subunit.

Lineweaver–Burk analyses for K_m and V_{max} of native enzyme and peak I (Figure 2) did not indicate any major differences. The normal enzyme had a K_m of 4.76×10^{-5} M for InGP; peak I had a K_m of 10.0×10^{-5} M. V_{max} values for the normal enzyme and peak I were identical.

The low level of activity in peak II fractions (0–10% that of the normal enzyme) is probably due to trailing of active peak I protein since on analytical polyacrylamide gels, peak II always contained trace amounts of peak I.

In summary, peak I is thought to contain (a) unlabeled protein, fully active by itself or in the presence of the β_2 subunit and (b) cysteine-80 labeled protein (Malkinson and Hardman, 1969) which has no activity by itself but about one-half the normal activity with the β_2 subunit. Peak II material is totally inactive.

Association of N-Ethylmaleimide-Treated α-Subunit Derivatives with the β_2 Subunit. The native α and β_2 subunits form a demonstratable physical complex, $\alpha_2\beta_2$, in the presence of pyridoxal phosphate and serine cofactors (Creighton and Yanofsky, 1966; Goldberg *et al.*, 1966). Under these conditions, equal amounts of both subunits form a 6.4S peak in 5–20% sucrose gradients. If either subunit is in excess, a peak corresponding to the apparent S of that subunit (2.7 S for the α subunit and 5.1 S for the β_2 subunit) is found in addition to the 6.4S peak. Sucrose gradient centrifugations of the *N*-ethylmaleimide-treated α-subunit derivatives in the absence of the β_2 subunit gave apparent S values corresponding to that of untreated protein (*i.e.*, a monomer of molecular weight of approximately 30,000).

When the unfractionated *N*-ethylmaleimide-labeled α subunit was sedimented through a 5–20% sucrose gradient in the presence of the β_2 subunit and cofactors, only a single peak of radioactivity was found. This peak corresponded to the position where the α subunit sediments by itself and contained only 31% of the radioactivity. The remainder of the radioactivity (69%) was recovered from the bottom of the centrifuge tube. No β_2-subunit enzymatic activity was found anywhere in the gradient implying that it had also pelleted to the bottom of the tube. In four separate experiments in which the

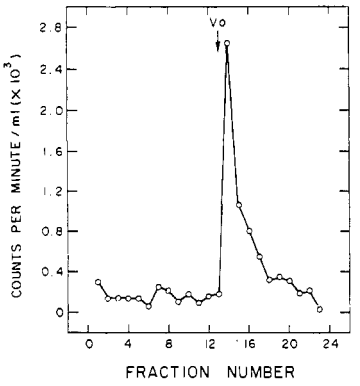

FIGURE 4: Sephadex G-200 gel filtration of peak II in the presence of the β_2 subunit. A 0.1-ml aliquot of peak II (*N*-ethylmaleimide/protein 1.43) was applied to a Sephadex G-200 column together with the β_2 subunit, pyridoxal phosphate, and L-serine and eluted as described in Methods. The radioactivity of 0.4-ml fractions was determined.

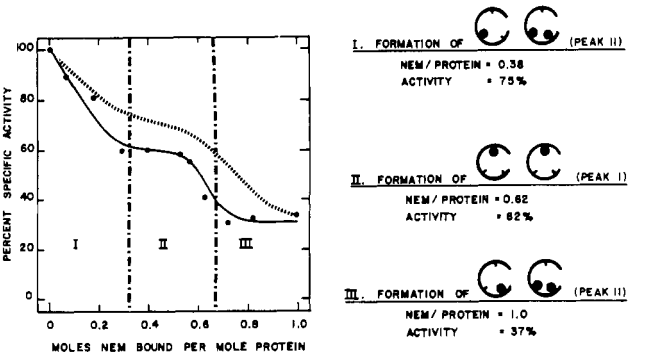

FIGURE 6: Diagrammatic representation of the α subunit inactivation by *N*-ethylmaleimide. The partly closed circles at the right of the figure refer to the α-subunit molecules labeled with *N*-ethylmaleimide, closed circles, at the different cysteine residues (see text and Figure 8, preceding paper). The calculations of *N*-ethylmaleimide/protein ratios and the relative specific activities are based on the relative amounts of the designated *N*-ethylmaleimide-labeled forms and the total population of molecules in unfractionated preparations (see Figure 8, preceding paper). The solid line in the figure repeats the data in Figure 5; the dotted line is the calculated curve.

ratio of the β_2 subunit/*N*-ethylmaleimide α subunit was varied from 0.5 to 5.0, the same observations were made (*i.e.*, about 70% of the radioactivity had pelleted and no β_2-subunit activity could be detected within the gradient).

A Sephadex G-200 filtration of unfractionated *N*-ethylmaleimide-treated protein, in the presence of equivalent levels of the β_2 subunit, resulted in the exclusion of 72% of the radioactivity from the gel and the total loss of any β_2-subunit activity.

That this behavior was due to the presence of peak II material in the unfractionated protein was confirmed by similar experiments with isolated peak I and peak II. With peak I protein in excess, both a 2.9S and a 6.1S peak were found in sucrose gradients (Figure 3), and all of the β_2-subunit enzymatic activity was contained in the 6.1S peak. Sucrose gradients of peak II with the β_2 subunit contained no radioactivity nor any β_2-subunit activity within the gradient. All of the radioactivity was at the bottom of the centrifuge tube. A Sephadex G-200 elution profile of peak II in the presence of the β_2 subunit (Figure 4) showed that all the radioactivity was excluded from the gel. Again no activity was recovered.

These results indicate that all of peak I combines normally with the β_2 subunit and implies that the cysteine-80-labeled protein in peak I can form the normal tetramer complex even

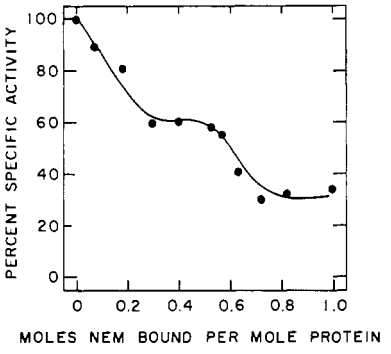

FIGURE 5: The relative specific activity (with a 10–20-fold excess of the β_2 subunit) of the *N*-ethylmaleimide-treated α subunit containing different *N*-ethylmaleimide/protein ratios. The In → Trp activity was measured.

though it appears to have substantially less activity than the unlabeled protein. Quantitative determinations of any differences between these forms in their rates of association or their association constants with the β_2 subunit (Creighton and Yanofsky, 1966) were not possible since they have not been separated.

The behavior of peak II on both sucrose gradients and Sephadex G-200 indicates that a large inactive aggregate is formed between peak II forms and the β_2 subunit. Molecular weight estimates as high as $0.5–1.0 \times 10^6$ indicate that the molar ratio of α subunit to β_2 subunit must be markedly different from the normal $\alpha_2\beta_2$ complex. Further, the relative affinity of peak II material for the β_2 subunit must be much greater than that of peak I since even fivefold molar excesses of the β_2 subunit can be preferentially aggregated by peak II present in unfractionated protein preparations.

Intermediate Formation of Active and Inactive N-Ethylmaleimide Derivatives of the α Subunit as a Function of N-Ethylmaleimide Bound. The problem of reconciling the relative amounts of active and inactive protein with the residual activity of either 0% (without the β_2 subunit) or 35–40% (with the β_2 subunit) was explored in the following way. The formation of the different *N*-ethylmaleimide-labeled forms was measured as a function of the amount of *N*-ethylmaleimide bound. Since no interactions of unlabeled and labeled protein were evident when activity measurements were carried out with the β_2 subunit, this assay was employed to detect the relative amounts of active and inactive enzyme formed during the labeling process. The α subunit was labeled to different extents by varying the amount of added *N*-ethylmaleimide as described before (Malkinson and Hardman, 1969). After removal of unreacted *N*-ethylmaleimide, the *N*-ethylmaleimide/protein ratios and In →Trp activities (with the β_2 subunit) of the samples were determined. The results are shown in Figure 5. During the initial binding (*N*-ethylmaleimide/protein 0–0.3), 35–40% of the activity is lost. No further loss occurs as an additional one-third mole of *N*-ethylmaleimide becomes bound (*N*-ethylmaleimide/protein 0.3–0.6). Finally, another

drop in activity to 35–40% is observed as the protein becomes fully labeled. These data suggest that inactive peak II material is being formed predominately at the low and high *N*-ethylmaleimide concentrations and partially active peak I labeled protein, at intermediate concentrations of *N*-ethylmaleimide.

Preliminary experiments on the fractionation of partially labeled preparations indicate that at low concentrations of *N*-ethylmaleimide, the predominant residue(s) being labeled are those which appear in peak II. For example, fractionation of the enzyme labeled to the extent of 0.22 mole of *N*-ethylmaleimide per mole of protein yields 80% peak I. Estimates of peak II protein are not meaningful since it was difficult to distinguish between the relative amounts of peak II and trailing peak I in the low amount of total protein (approximately 20%) eluting at this position. More important, however, is the fact that in peak I, where these problems are much less significant, the *N*-ethylmaleimide/protein ratio is 0.08. This would indicate that there is only about 15% of the maximum labeling of cysteine-80 (peak I), the majority of labeling occurring at cysteine-117 and/or cysteine-153 (peak II).

The simplest interpretation of all the enzymatic and chemical data presented in this and the preceding paper is shown diagrammatically in Figure 6. As mentioned above, the formation of peak II material is postulated to occur at the initial (stage I) and final (stage III) stages of *N*-ethylmaleimide labeling under these conditions. Whether cysteine-117 or cysteine-153 is labeled initially is not known although it would matter little in this interpretation since each is inactive. Simple calculations of *N*-ethylmaleimide/protein ratios and enzymatic activity expected at the end of each stage are presented at the right of the figure. The dotted line in the figure represents the theoretical curve obtained from such calculations if there was no overlapping of the different stages of *N*-ethylmaleimide labeling. Some overlapping of stages I and II and stages II and III would shift this curve lower and to the left. The preliminary evidence suggests some overlapping of stages I and II, at least. Association between unlabeled protein and peak II protein (during both the initial and final stages) could lead to masking of the *N*-ethylmaleimide reactivity and the enzymatic activity, alone, of the unlabeled protein and result in a linear relationship between *N*-ethylmaleimide labeling and the loss of this activity. More conclusive evidence for this model will come from a determination of the relative amounts of the different labeled tryptic peptides found at various levels of labeling.

Discussion

The correlation between the structural and functional roles of the three cysteine residues in the α subunit can be summarized as follows. Labeling of cysteine-80 leads to little detectable alterations in its physical association with the antibody or the β_2 subunit. Enzymatically, this protein appears to be totally inactive by itself and about 50% active in combination with the β_2 subunit. Labeling of either cysteine-117 or cysteine-153 results in major structural and functional changes in the protein: additional *N*-ethylmaleimide reactivity at either cysteine-153 or cysteine-117 to yield doubly labeled protein; a complete loss of antibody recognition and enzymatic activity; and a totally anomalous association with the β_2 subunit.

Although the evidence for the proposed model of the *N*-ethylmaleimide inactivation of the α subunit is largely indirect, it can account for most of the chemical and enzymatic data. Further speculation regarding why *N*-ethylmaleimide reacts in this way would be pointless until more direct evidence is available to verify this proposal.

Acknowledgment

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References

Crawford, I. P., and Yanofsky, C. (1959), *Proc. Natl. Acad. Sci. U. S. 45*, 1280.

Creighton, T. E., and Yanofsky, C. (1966), *J. Biol. Chem. 241*, 980.

Creighton, T. E., and Yanofsky, C. (1969), *Methods Enzymol.* (in press).

Goldberg, M. E., Creighton, T. E., Baldwin, R. L., and Yanofsky, C. (1966), *J. Mol. Biol. 21*, 71.

Hardman, J. K., and Yanofsky, C. (1965), *J. Biol. Chem. 240*, 725.

Hatanaka, M., White, E. A., Horibata, K., and Crawford, I. P. (1962), *Arch. Biochem. Biophys. 97*, 596.

Malkinson, A. M., and Hardman, J. K. (1969), *Biochemistry 8*, 2769 (this issue; paper I).

Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Smith, O., and Yanofsky, C. (1962), *Methods Enzymol. 5*, 794.

Yanofsky, C. (1956), *J. Biol. Chem. 223*, 171.