Reaction of Phenylglyoxal and (*p*-Hydroxyphenyl)glyoxal with Arginines and Cysteines in the α Subunit of Tryptophan Synthase†

Hyone-Myong Eun* and Edith Wilson Miles‡

ABSTRACT: The α subunit of tryptophan synthase from *Escherichia coli* is inactivated by phenylglyoxal and by (*p*-hydroxyphenyl)glyoxal. The use of these chemical modification reagents to determine the role of arginyl residues in the α subunit of tryptophan synthase has been complicated by our finding that these reagents react with sulfhydryl groups of the α subunit, as well as with arginyl residues. Analyses of the data for incorporation of phenyl[2-^{14}C]glyoxal, for inactivation, and for sulfhydryl modification in the presence and absence of indole-3-glycerol phosphate indicate that two sulfhydryl groups and one arginine are essential for the activity. Our finding that the substrate protects the single essential arginyl residue but not the two sulfhydryl groups is consistent with the observed kinetics of partial protection by substrate or by a substrate analogue, indole-3-propanol phosphate. In contrast to phenylglyoxal, (*p*-hydroxyphenyl)glyoxal modifies two to three sulhydryl groups that are not protected by indole-3-glycerol phosphate and modifies none of the arginyl residues that are modified by phenylglyoxal.

Although extensive studies have been made of the genetics, sequence, and evolution of the tryptophan operon (Yanofsky et al., 1981; Platt, 1978) and of the interaction of two of its gene products to form the $\alpha_2\beta_2$ complex of tryptophan synthase [for reviews, see Yanofsky & Crawford (1972) and Miles (1979)], very little is known about the active site of the α subunit and the residues involved in substrate binding and catalysis.

The α subunit catalyzes the aldolytic conversion of indole-3-glycerol phosphate to indole and D-glyceraldehyde 3-phosphate (reaction 1). Free indole combines with L-serine at the active site of the β_2 subunit to form L-tryptophan (reaction 2). The sum of reaction 1 and reaction 2 is reaction 3, the physiologically important reaction catalyzed by the $\alpha_2\beta_2$ complex:

indole-3-glycerol phosphate $\rightleftarrows$ indole + D-glyceraldehyde 3-phosphate (1)

indole + L-serine $\rightarrow$ L-tryptophan + H_2O (2)

indole-3-glycerol phosphate + L-serine $\rightarrow$ L-tryptophan + D-glyceraldehyde 3-phosphate + H_2O (3)

Although the α and β_2 subunits are capable of carrying out the independent reactions 1 and 2, respectively, the catalytic efficiency and reaction specificity are significantly modulated by subunit interactions (Miles, 1979; Tschopp & Kirschner, 1980; Kirschner & Wiskocil, 1972).

In the present study, we have investigated the role of arginyl residues in the indole-3-glycerol phosphate binding site of the α subunit by using phenylglyoxal, which is a useful reagent for the modification of arginyl residues in the anion binding sites of enzymes (Takahashi, 1968, 1977; Riordan et al., 1977) (see Discussion). Phenylglyoxal has been used in this laboratory to identify the essential arginine–148 of the β_2 subunit of tryptophan synthase (Tanizawa & Miles, 1983). During the course of our studies using chemical modification by phenylglyoxal (Takahashi, 1968, 1977) and by (*p*-hydroxyphenyl)glyoxal (Yamasaki et al., 1980), both of which are generally thought to be arginine specific under mild conditions, we have found that these reagents also react with sulfhydryl groups in several enzymes. Since modification of sulfhydryl groups in the α subunit causes inactivation, as previously shown with other sulfhydryl reagents (Hardman & Yanofsky, 1965; Malkinson & Hardman, 1969), and occurs concomitantly with modification of arginyl residues by phenylglyoxal, it is difficult to assess whether the arginine is essential. However, through comparative analysis of the effects of total incorporation of the reagents and of the specific modification of sulfhydryl groups upon inactivation, we conclude that phenylglyoxal reacts with one essential arginine in addition to two essential sulfhydryl groups in the α subunit of *Escherichia coli* tryptophan synthase.

Materials and Methods

Materials. Phenyl[2-^{14}C]glyoxal (32 mCi/mmol) (Research Products International Corp.), L-serine (Sigma), pyridoxal phosphate (Sigma), dithiothreitol (Bethesda Research Laboratories or Calbiochem), (*p*-hydroxyphenyl)glyoxal (monohydrate) (Pierce), β-nicotinamide adenine dinucleotide (Sigma), urea (ultrapure) (Bethesda Research Laboratories), glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle) (Sigma), and Sephadex G-25 (medium, fine, superfine) (Pharmacia) were commercial products.

Phenylglyoxal (monohydrate) (Aldrich) was recrystallized from hot distilled water (mp 78.2–79.5 °C). Indole-3-propanol phosphate was a generous gift of Dr. K. Kirschner. Indole-3-glycerol phosphate was synthesized enzymatically from indole and fructose 1,6-diphosphate plus aldolase (Worthington) and the $\alpha_2\beta_2$ complex of tryptophan synthase (Hardman & Yanofsky, 1965).

Enzymes and Enzyme Assays. The tryptophan synthase $\alpha_2\beta_2$ complex was prepared as described by Adachi et al. (1974) from *E. coli* strain W3110 *trpRcysBtrpED102trpB*$^+$-*trpA*$^+$/*F'colVBcysB*$^+$*trpED102trpB*$^+$*trpA*$^+$.[1] The α and β_2 subunits were prepared from the $\alpha_2\beta_2$ complex as described

† From the Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Building 4, Room 116, Bethesda, Maryland 20205. *Received April 6, 1984.* A preliminary account of this work was presented at the Annual Meeting of the American Society of Biological Chemists, St. Louis MO, June 4–7, 1984 (Miles & Eun, 1984).

* Address correspondence to this author at the Department of Microbiology and Infectious Diseases, Health Science Centre, University of Calgary, N.W. Calgary, Alberta, Canada T2N 4N1.

‡ Address requests for reprints to this author.

[1] We thank Drs. C. Yanofsky and I. P. Crawford for gifts of the *E. coli* strain.

0006-2960/84/0423-6484$01.50/0

by Miles & Moriguchi (1977) except that the Sephadex G-100 superfine column was replaced by a Sephacryl S-200 superfine column (3 cm × 90 cm) in order to increase the flow rate. The α subunit from *Salmonella typhimurium* was a gift of Dr. S. A. Ahmed.[2] The β_2 subunit was further purified by heat treatment for 3 min at 80 °C, which is a slight modification of the procedure of Wilson & Crawford (1965).

The activity of each subunit was assayed at 37 °C in the presence of an approximately 3-fold excess of the complementary subunit. The α specific activity (reaction 1) was assayed spectrophotometrically by using the glyceraldehyde-3-phosphate dehydrogenase coupled reaction as described by Creighton & Yanofsky (1966). The assay mixture also contained 50 mM sodium borate, pH 7.8. The β specific activity (reaction 2) was assayed by following the procedure described by Higgins et al. (1979). Protein concentrations were determined from the specific absorbance of the α subunit ($E^{1\%}_{278}$ = 4.4) (Adachi et al., 1974) and of the holo-β_2 subunit ($E^{1\%}_{278}$ = 6.5) (Hathaway & Crawford, 1970). Protein concentrations of modified proteins were determined by the method of Lowry et al. (1951) with unmodified protein as a standard.

Chemical Modification Reactions. The stock solution of phenylglyoxal (0.1 M) was prepared by first dissolving the crystals in absolute methanol and then diluting the solution with 4 volumes of water. The exact concentration of phenylglyoxal was determined by the absorbance at 247 nm (ϵ = 11 300 M^{-1} cm^{-1} in methanolic solution) (Kohlbrenner & Cross, 1978). A 0.1 M stock solution of (*p*-hydroxyphenyl)glyoxal was prepared directly in water. The reagent solutions were stable for at least 1 month when stored at −20 °C in the dark.

The modification reactions were carried out at 30 °C in a medium containing 50 mM borate (pH 7.8), which stabilizes a 1:1 complex between phenylglyoxal and arginine (Borders & Riordan, 1975; Takahashi, 1977). Borate has not previously been used with (*p*-hydroxyphenyl)glyoxal (Yamasaki et al., 1980; see Discussion). The reactions were performed in the dark to avoid potential photochemical side reactions (Mäkinen et al., 1982). A typical reaction mixture contained 50 mM triethanolamine buffer, pH 7.8, 50 mM sodium borate, 1.0–1.8 mg/mL α subunit, and 10 mM phenylglyoxal or (*p*-hydroxyphenyl)glyoxal. Aliquots (usually 195 μL) were taken at time intervals and applied to columns of Sephadex G-25 (fine or medium) (see below). The columns were suspended in glass centrifuge tubes and centrifuged at 300 rpm for 2 min at room temperature in a desk-top centrifuge. This fast gel filtration method achieves nearly 100% elimination of the excess reagents. The filtered enzyme was usually obtained in 90% yield and was used for assay of residual activity and for the determination of the extent of modification. Since we initially demonstrated that the amount of protein in each filtrate as determined by the method of Lowry et al. (1951) was highly reproducible (greater than 99%), we subsequently determined the protein concentration in each filtrate from the activity of the unmodified enzyme in the filtrate and from the specific activity of the unmodified enzyme prior to filtration.

The columns of Sephadex G-25 were prepared in conical, plastic pipet tips (1 mL) (Rainin) plugged with glass wool to give a bed height of 5.5 cm after centrifugation as above. The columns were preequilibrated by addition of 0.2 mL of 0.1 M sodium phosphate buffer, pH 7.0, followed by centrifugation as above; this was repeated 3 times. Many variations of this method have been used by other authors [see, for example, Tuszynski et al., (1980)].

Determination of Extent of Modification. The available sulfhydryl groups were titrated at 37 °C with 5,5′-dithiobis-(2-nitrobenzoic acid) (0.2 mM final concentration) in 0.1 M sodium phosphate buffer, pH 7.0, containing 6 M urea. The concentration of sulfhydryl groups was calculated by using ϵ_{412nm} = 13.6 mM^{-1} cm^{-1} [Ellman, 1959; also see refined values in Riddles et al. (1983)].

The extent of (*p*-hydroxyphenyl)glyoxal incorporation was determined by the absorbance at 340 nm at pH 9.0 (0.1 M sodium pyrophosphate buffer) with ϵ = 18.3 mM^{-1} cm^{-1}, which was originally estimated for the arginine adduct in the absence of borate (Yamasaki et al., 1980). We used the same molar absorption coefficient, since the thiol (*N*-acetyl-L-cysteine) adduct gave similar molar absorption properties at 340 nm, although the λ_{max} showed a slight bathochromic shift to 343 nm[3] (see Discussion).

The incorporation of phenylglyoxal into protein was determined by using phenyl[2-^{14}C]glyoxal (specific radioactivity = 1.62 × 10^3 cpm/nmol). A 100-μL gel-filtered sample (≃2 nmol of α subunit) was counted in 10 mL of Aquasol (New England Nuclear) in a Beckman LS-8100 scintillation counter.

Analysis of Data. The number of essential residues was determined by the statistical analysis of Tsou (1962) from the number of groups modified and the residual activity of partially modified protein. Tsou's method treats the cases where all of the same type of groups react at the same rate with a modifying reagent, with the assumption that modification of any one of the essential groups leads to complete loss of activity. The general relationship of the fraction of the remaining activity *a* is given by $a^{1/i} = (p - m)/p$, where *m* is the number of modified groups and *p* is the total number of modifiable groups in a protein among which only *i* are essential. This relationship also holds when there are two or more types of groups that are modified at similar rates by the same reagent. The number of essential groups *i*, normally a small integer, is found from the best straight line of the plot $a^{1/i}$ vs. *m*.

Results

The α Subunit of Tryptophan Synthase Is Inactivated by Phenylglyoxal and (p-Hydroxyphenyl)glyoxal. The rate of inactivation of the α subunit by excess phenylglyoxal follows pseudo-first-order kinetics (Figure 1A). The inactivation of the α subunit by (*p*-hydroxyphenyl)glyoxal proceeds in a similar fashion, although at a slightly slower rate. The plot of the reciprocal first-order rate constants ($1/k_{obsd}$) vs. 1/[phenylglyoxal] or 1/[(*p*-hydroxyphenyl)glyoxal] gives a straight line that intersects the ordinate (Figure 1B). These data indicate that the inactivator (I) forms a reversible noncovalent enzyme–reagent complex (EI) prior to irreversible modification and inactivation (EI*) (Kitz & Wilson, 1962):

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^* \qquad (1)$$

The dissociation constants K_I for both phenylglyoxal and (*p*-hydroxyphenyl)glyoxal can be calculated from the intercept on the abscissa (*x* axis) to be 33 mM. The rate constants of inactivation (k_3) by phenylglyoxal and (*p*-hydroxyphenyl)-glyoxal are 0.27 min^{-1} and 0.21 min^{-1}, respectively, at pH 7.8 and 30 °C. The rates of inactivation (k_3) of the holo-$\alpha_2\beta_2$ complex by phenylglyoxal and (*p*-hydroxyphenyl)glyoxal are 6- and 4-fold slower, respectively, than the rates of inactivation of the α subunit by these reagents (data not shown).

[2] We thank Dr. S. A. Ahmed for a gift of the α subunit of tryptophan synthase from *S. typhimurium* (S. A. Ahmed, T. Fairwell, K. Yutani, R. Bauerle, and E. W. Miles, unpublished results).

[3] H.-M. Eun, unpublished results.

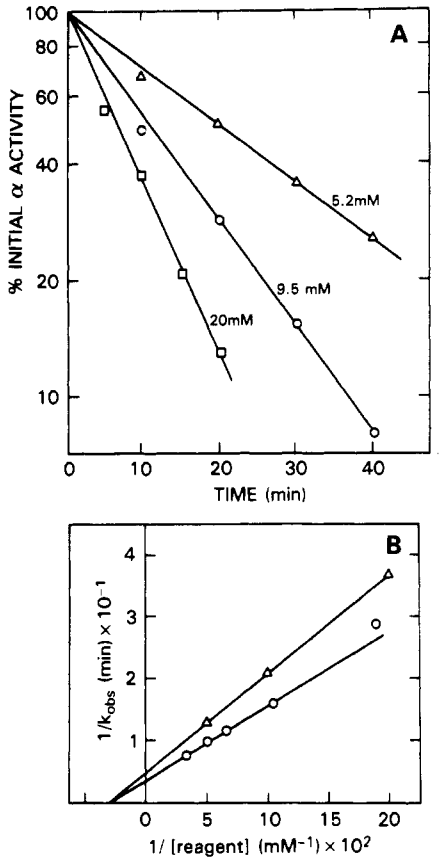
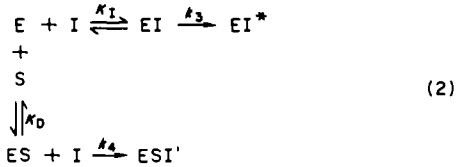


FIGURE 1: Effect of phenylglyoxal or (*p*-hydroxyphenyl)glyoxal concentration upon the rate of inactivation. α subunit (0.52 mg/mL) was incubated with varying concentrations of the modifying reagents as described under Materials and Methods. Aliquots of 45 μL were added to 105 μL of L-arginine (0.1 M, pH 9.0) at 0 °C to quench the excess reagents and then filtered through Sephadex G-25 centrifuge columns. The residual activity was assayed as described under Materials and Methods. The control retains full activity over this period of time. (A) Plot of the percent initial α activity (on a semilogarithmic scale) vs. incubation time (min) at three concentrations of phenylglyoxal: 5.2 (△), 9.5 (○), and 20 mM (□). Similar plots for the modification by (*p*-hydroxyphenyl)glyoxal are not shown. (B) The reciprocal plot of the first -order rate constants of inactivation (k_{obsd}) obtained from (A) and other plots not shown vs. the reagent concentration: phenylglyoxal (○) and (*p*-hydroxyphenyl)glyoxal (△).

Protection of the α Subunit by Indole-3-glycerol Phosphate and Indole-3-propanol Phosphate. The substrate indole-3-glycerol phosphate and the competitive inhibitor indole-3-propanol phosphate decrease the rate of inactivation of the α subunit by phenylglyoxal and (*p*-hydroxyphenyl)glyoxal. Indole-3-glycerol phosphate and indole-3-propanol phosphate demonstrate a specific concentration-dependent protection at low substrate (or inhibitor) concentrations but no further protection at high concentrations (Figure 2). These results imply that the inactivation of the α subunit by phenylglyoxal or (*p*-hydroxyphenyl)glyoxal originates from reactions at two different sites: one essential site that can be protected by indole-3-glycerol phosphate or indole-3-propanol phosphate and another essential site that cannot be protected by indole-3-glycerol phosphate or indole-3-propanol phosphate. The minimal mechanism that is consistent with the kinetics of inactivation and substrate protection is described by eq 2,



(2)

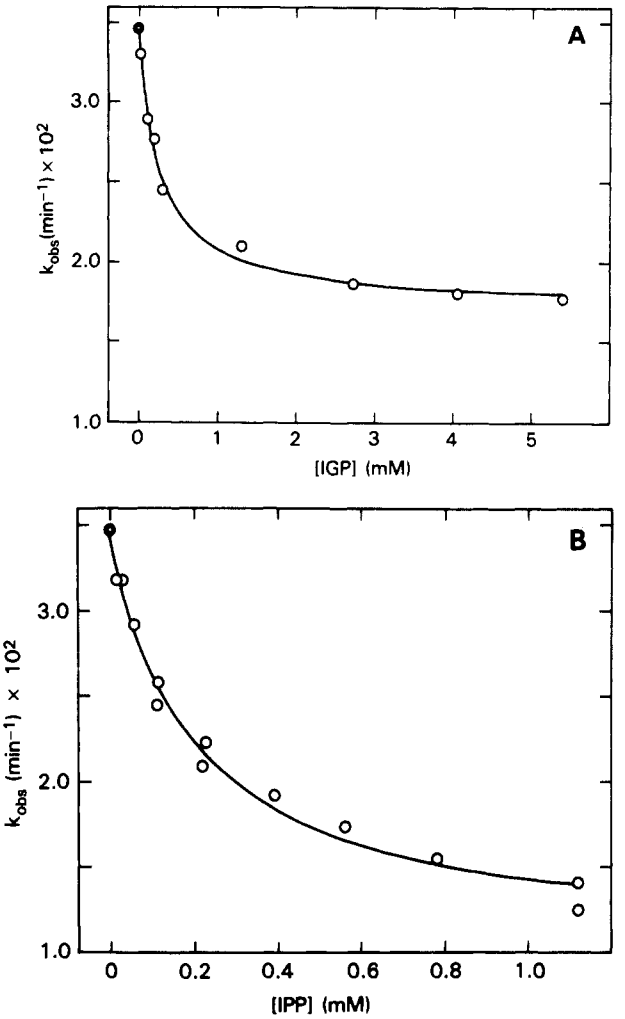


FIGURE 2: Effect of indole-3-glycerol phosphate and indole-3-propanol phosphate concentration upon inactivation of the α subunit by phenylglyoxal or (*p*-hydroxyphenyl)glyoxal. α subunit (0.72 mg/mL) was incubated at pH 7.8 and 30 °C with 10 mM (*p*-hydroxyphenyl)glyoxal in the presence of indole-3-glycerol phosphate (A) or with 10 mM phenylglyoxal in the presence of indole-3-propanol phosphate (B). Aliquots were taken at intervals, submitted to gel filtration, and assayed as described under Materials and Methods. The first-order rate constants (k_{obsd}) of inactivation are plotted as a function of indole-3-glycerol phosphate or indole-3-propanol phosphate concentration. The solid line in (A) is the theoretical plot according to eq 3 with the parameters given in Table I. Under the conditions of incubation at high indole-3-glycerol phosphate concentrations, e.g., 5.4 mM, the amount of catalytic cleavage of the substrate is estimated to be below 3%. The solid line in (B) is the theoretical plot according to eq 3 with the parameters given in Table I. IGP, indole-3-glycerol phosphate; IPP, indole-3-propanol phosphate.

where the free enzyme (E) can react with the modifying reagent (I) to form an inactive complex (EI*, as in eq 1) or where the enzyme first forms complex (ES) with the substrate or the substrate analogue and the ES complex can still react with the reagent (I) to form an inactive complex (ESI'). This mechanism (eq 2) assumes that the rate of inactivation is reduced as a result of the substrate (or analogue) binding at the active site of the enzyme. The observed rate constant (k_{obsd}) of inactivation is related (see Appendix) to the concentrations of the modifying reagent and the substrate by eq 3. The kinetic parameters K_I, K_D, k_3, and k_4 were estimated

$$k_{obsd} = \frac{k_3([I]/K_i) + k_4[I]([S]/K_D)}{1 + [I]/K_I + [S]/K_D} \qquad (3)$$

from the best fit of the data to eq 3 (Table I). The K_I value of 34 mM for both phenylglyoxal and (*p*-hydroxyphenyl)-

Table I: Kinetics of Inactivation of α Subunit Modified by Phenylglyoxal and (*p*-Hydroxyphenyl)glyoxal in the Presence of Indole-3-glycerol Phosphate or Indole-3-propanol Phosphate[a]

parameters	phenylglyoxal	(*p*-hydroxyphenyl)glyoxal
K_I (mM)	33.7	33.9
k_3 (min^{-1})	0.15	0.15
k_4 (M^{-1} min^{-1})	1.01	1.70
K_D for indole-3-glycerol phosphate (mM)		0.21
K_D for indole-3-propanol phosphate (mM)		0.16

[a] The kinetic parameters were determined by iterative curve fittings of the data in Figure 2 to eq 3 with MLAB program of the National Institutes of Health.

glyoxal agrees well with the previous results from the inactivation kinetics (eq 1) in the absence of the effectors. The estimated dissociation constants (K_D) of 0.21 mM and 0.16 mM for indole-3-glycerol phosphate and indole-3-propanol phosphate, respectively, are also of the same magnitude as the reported values of 0.48 mM for indole-3-glycerol phosphate (K_m) (Weischet & Kirschner, 1976) and 0.05–0.1 mM for indole-3-propanol phosphate (K_D) (Heyn & Weischet, 1975). Thus, our results strongly support the model of inactivation kinetics as described by eq 2.

Phenylglyoxal Inactivation of the α Subunit Involves Three Essential Residues. The plot of the residual activity vs. the number of modified residues is not linear (Figure 3A). Complete loss of activity is related to the modification of five to six residues. When the data are analyzed by the statistical method of Tsou (1962), the best straight line is obtained when we assume that there are three essential residues (i.e., $i = 3$).

When the inactivation experiment was repeated in the presence of the substrate indole-3-glycerol phosphate (1.1 and 2.7 mM), the plot of the residual activity vs. the number of modified residues is curved when $i = 1$ but gives a good straight line fit when $i = 2$ (Figure 3A). Thus, indole-3-glycerol phosphate protects one essential residue from modification by phenylglyoxal. The finding that two other essential residues react with phenylglyoxal in the presence of near saturating concentrations of indole-3-glycerol phosphate is consistent with the observation in Figure 2 that indole-3-glycerol phosphate or indole-3-propanol phosphate only partially protects the α subunit. The evidence that these two other essential residues correspond to the sulfhydryl groups is presented later. In addition to the three essential groups, phenylglyoxal apparently modifies two or three nonessential residues of the α subunit.

Inactivation of the α Subunit by (p-Hydroxyphenyl)glyoxal. The plot of the residual α activity vs. the number of residues modifed by (*p*-hydroxyphenyl)glyoxal is linear when $i = 1$ (Figure 4). Although the total number of modifiable residues is two to three, complete loss of activity apparently results from the modification of a single essential residue. While indole-3-glycerol phosphate (2.1 mM) does significantly decrease the rate of inactivation (see Figure 2A), it does not protect an essential residue (Figure 4).

Modification of Essential Sulfhydryl Groups by Phenylglyoxal and (p-Hydroxyphenyl)glyoxal. Inactivation of the α subunit by phenylglyoxal and (*p*-hydroxyphenyl)glyoxal is correlated with a decrease in the sulfhydryl content (Figures 3B and 4 and Table II). The presence of indole-3-glycerol phosphate during modification has no effect on the extent of sulfhydryl modification. The data for inactivation by phenylglyoxal give a best straight line fit (Tsou, 1962) for two essential sulfhydryl groups, whether in the presence or in the absence of the substrate indole-3-glycerol phosphate (Figure

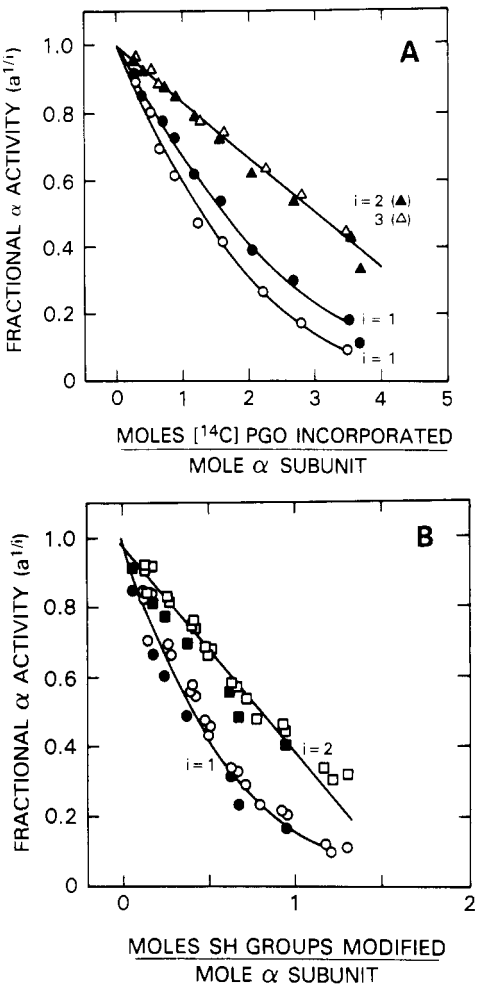


FIGURE 3: Effect of extent of incorporation (A) and of sulfhydryl modification (B) by phenylglyoxal upon activity. (A) Plots of the residual α activity vs. moles of phenyl[2-^{14}C]glyoxal incorporated per mole of α subunit in the absence (○) and presence (●) of indole-3-glycerol phosphate when the number of essential residue was $i = 1$ (see Materials and Methods), in the absence of indole-3-glycerol phosphate when $i = 3$ (△), and in the presence of indole-3-glycerol phosphate when $i = 2$ (▲). α subunit (2.12 mg/mL) in a reaction volume of 500 μL was incubated at 30 °C with 6.6 mM phenyl[2-^{14}C]glyoxal (specific radioactivity = 1.62 10^3 cpm/nmol) in 60 mM triethanolamine buffer, pH 7.8, containing 60 mM sodium borate in the absence and presence of 1.1 mM indole-3-glycerol phosphate. Aliquots (50 μL) were removed at intervals, passed through Sephadex G-25 superfine centrifuge columns that were preequilibrated in 0.1 M sodium borate, pH 7.8, and counted as described under Materials and Methods. The incubation in the presence of 2.7 mM indole-3-glycerol phosphate (data not shown) gives similar results as that in the presence of 1.1 mM indole-3-glycerol phosphate. (B) Plots of the residual α activity vs. moles of sulfhydryl groups modified per mole of α subunit in the absence (○) and presence (●) of indole-3-glycerol phosphate when $i = 1$ and in the absence (□) and presence (■) of indole-3-glycerol phosphate when $i = 2$. α subunit (1.78 mg/mL) was incubated with 10 mM phenylglyoxal in the absence and presence of 2.1 mM indole-3-glycerol phosphate. Aliquots of 195 μL were taken at time intervals, passed through Sephadex G-25 fine centrifuge columns, and then used for the residual activity assay and for the sulfhydryl titration by 5,5′-dithiobis(2-nitrobenzoic acid) in 6 M urea (see Materials and Methods). SH, sulfhydryl group; PGO, phenylglyoxal.

3B). Since the phenylglyoxal incorporation studies (Figure 3A) indicate the presence of a third essential residue that is protected by indole-3-glycerol phosphate, this third essential residue must be a different amino acid, most probably arginine. Since our data for total incorporation of (*p*-hydroxyphenyl)-glyoxal and sulfhydryl disappearance (Figure 4) are practically identical, (*p*-hydroxyphenyl)glyoxal appears to react only with sulfhydryl groups in the α subunit and not with arginine.

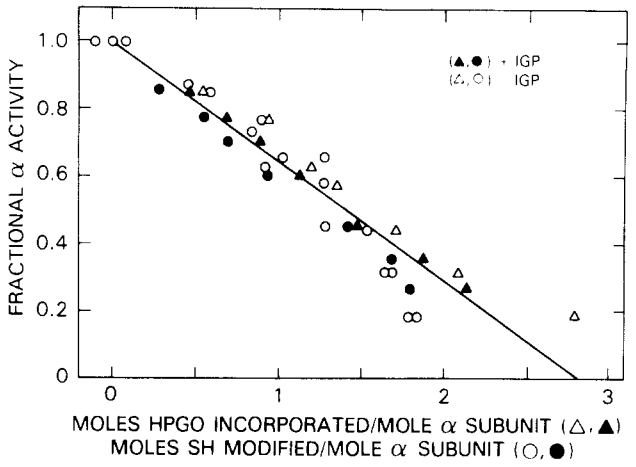


FIGURE 4: Effect of extent of (*p*-hydroxyphenyl)glyoxal incorporation and of sulfhydryl modification upon activity. α subunit (1.78 mg/mL) was incubated with 10 mM (*p*-hydroxyphenyl)glyoxal in the absence and presence of 2.1 mM indole-3-glycerol phosphate. Aliquots of the incubation mixture were passed through Sephadex G-25 fine centrifuge columns that were preequilibrated with 0.1 M sodium pyrophosphate, pH 9.0. The extent of (*p*-hydroxyphenyl)glyoxal incorporation was determined from the absorbance at 340 nm at room temperature as described under Materials and Methods. The 5,5′-dithiobis(2-nitrobenzoic acid) titration was carried out in 6 M urea at 37 °C by monitoring the absorbance change at 412 nm. (*p*-Hydroxyphenyl)-glyoxal incorporation in the absence (△) and presence (▲) of indole-3-glycerol phosphate; disappearance of sulfhydryl groups in the absence (○) and presence (●) of indole-3-glycerol phosphate. HPGO, (*p*-hydroxyphenyl)glyoxal; SH, sulfhydryl group; IGP, indole-3-glycerol phosphate.

(p-Hydroxyphenyl)glyoxal Modification of Sulfhydryl Groups of E. coli α Subunit Results in Conformational Changes. When the α subunit is incubated with either phenylglyoxal or (*p*-hydroxyphenyl)glyoxal, the first-order rate of inactivation in reaction 2 (the synthesis of tryptophan from indole and L-serine) is 2–3 times slower (data not shown) than the first-order rate of activity loss in reaction 1 (the cleavage of indole-3-glycerol phosphate) (Figure 1). This result suggests that modification of the α subunit impairs its ability to interact with the β_2 subunit.

Comparative Reactivities of Phenylglyoxal and (p-Hydroxyphenyl)glyoxal toward Sulfhydryl Groups. Since we made the unexpected finding that phenylglyoxal and (*p*-hydroxyphenyl)glyoxal react with sulfhydryl groups of the α subunit, we have also examined the reaction of these compounds with several proteins. As shown in Table II, phenylglyoxal and (*p*-hydroxyphenyl)glyoxal both react with sulfhydryl groups in the α subunits from *E. coli* and from *S. typhimurium*. However, only (*p*-hydroxyphenyl)glyoxal reacts with sulfhydryl groups in the apo-β_2 subunit of *E. coli* tryptophan synthase and in glyceraldehyde-3-phosphate dehydrogenase, whereas neither reagent reacts with the single accessible sulfhydryl group in the holo-β_2 subunit. Thus phenylglyoxal and (*p*-hydroxyphenyl)glyoxal can both react with sulfhydryl groups in some proteins but differ in their reactivity and in specificity with different protein sulfhydryls. (*p*-Hydroxyphenyl)glyoxal appears to be more reactive with sulfhydryls than does phenylglyoxal.

Table II: Reactions of Phenylglyoxal and (*p*-Hydroxyphenyl)glyoxal with Sulfhydryl Groups in Proteins

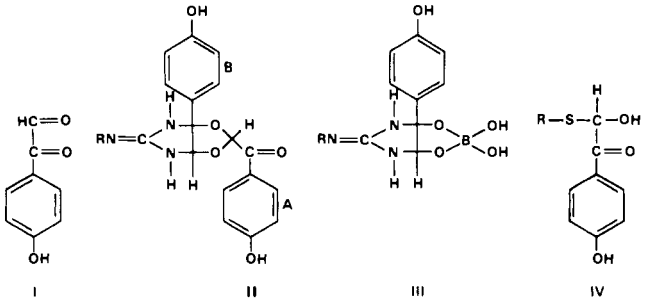
proteins	sulfhydryl groups modified/monomer: phenylglyoxal	sulfhydryl groups modified/monomer: (*p*-hydroxyphenyl)-glyoxal	sulfhydryl groups/monomer: found (control)	sulfhydryl groups/monomer: lit.	ref
tryptophan synthase (*E. coli*)					
α subunit	2	2–3	3.0 ± 0.2	3	*e*
holo-β_2 subunit[a]	0	0	5.1 ± 0.4	5	*f, g*
apo-β_2 subunit[b]	0	1.1–1.5	5.0 ± 0.1	5	*f, g*
tryptophan synthase (*S. typhimurium*)					
α subunit[c]	1–2	2–3	3.2 ± 0.4	3	*h*
glyceraldehyde-3-phosphate dehydrogenase[d]	0.1–0.2	0.7–0.8	3.6 ± 0.1	4	*i*

[a] The holo-β_2 subunit (2.4 mg/mL) was incubated with 10 mM phenylglyoxal or (*p*-hydroxyphenyl)glyoxal under conditions similar to those used for the *E. coli* α subunit (see Materials and Methods). The available sulfhydryl groups were determined as described under Materials and Methods with samples (2–4 nmol of β monomer) taken at 30- and 60-min incubation times. [b] The apo-β_2 subunit was prepared from holo-β_2 subunit by dialysis for 4 h against 100 volumes of 50 mM triethanolamine buffer, pH 7.8, containing 1 mM dithiothreitol, 2 mM EDTA, and 10 mM NH_2OH with one buffer change and was then dialyzed for 5 h against 100 volumes of 0.1 M phosphate, pH 7.0, with one buffer change. The apo-β_2 subunit (1.5 mg/mL) was incubated with 10 mM phenylglyoxal or (*p*-hydroxyphenyl)glyoxal and titrated with 5,5′-dithiobis(2-nitrobenzoic acid) as described for the holo-β_2 subunit (see footnote *a*). [c] The α subunit of *S. typhimurium* (1.75 mg/mL) was incubated with 10 mM phenylglyoxal or (*p*-hydroxyphenyl)glyoxal and analyzed for the extent of sulfhydryl group modification (see footnote *a*). [d] The crystalline suspension of glyceraldehyde-3-phosphate dehydrogenase in 2.6 M $(NH_4)_2SO_4$, pH 7.5, was diluted in 6 volumes of activation buffer composed of 50 mM triethanolamine, pH 7.8, 5 mM dithiothreitol, and 5 mM EDTA, incubated for 1 h at 0 °C, and then dialyzed overnight against 100 volumes of triethanolamine buffer (50 mM, pH 7.8) with one buffer change. Glyceraldehyde-3-phosphate dehydrogenase (3.4 mg/mL) was incubated with 10 mM phenylglyoxal or (*p*-hydroxyphenyl)glyoxal and analyzed for sulfhydryl group content (see footnote *a*). [e] Guest et al. (1967). [f] Miles & Higgins (1980). [g] Crawford et al. (1980). [h] Li & Yanofsky (1973). [i] Harris & Perham (1965).

Discussion

Studies with phenylglyoxal and similar reagents containing vicinal dicarbonyl groups (e.g., butanedione and cyclohexanedione) have shown that arginyl residues in the active sites of many proteins bind anionic substrates and cofactors (Riordan et al., 1977; Borders & Riordan, 1975; Patthy & Thész, 1980; Takahashi, 1968, 1977; Tanizawa & Miles, 1983). Since these active site arginyl residues are often unusually reactive with dicarbonyl reagents, Patthy & Thész (1980) have proposed that the charge potential of anion binding sites may lower the pK_a value of an arginine in the site and thus make the arginine more reactive chemically. Although rather low reagent concentrations have been used to modify highly reactive arginyl residues [e.g., 0.2 mM phenylglyoxal (Borders & Riordan, 1975) and 0.37 mM phenylglyoxal (Vandenbunder et al., 1981)], much higher concentrations have been used in studies of less reactive arginyl residues [e.g., 5–50 mM phenylglyoxal (Peters et al., 1981) and 5–40 mM phenylglyoxal (Kuno et al., 1980)]. Although these reagents are generally highly specific for arginyl residues, side reactions may occur, especially at higher reagent concentrations. Phenylglyoxal and other dicarbonyl reagents also react slowly with lysyl residues and rapidly deaminate α-amino groups of proteins (Takahashi, 1968, 1977). Phenylglyoxal forms a hemithioacetal with free sulfhydryl groups (Schubert, 1936), promotes formation of disulfide bond in rhodanese (Weng et al., 1978), and reacts with at least one sulfhydryl in serine hydroxymethyl transferase (Gavilanes et al., 1982). Other reported side reactions of dicarbonyl reagents include photodestruction of numerous residues by 2,3-butanedione (Mäkinen et al., 1982) and the modification of lysine by 2,4-pentanedione (Gilbert & O'Leary, 1975). (*p*-Nitro-

Chart I



phenyl)glyoxal also reacts with histidine and with sulfhydryl residues (Yamasaki et al., 1981). Our results show that phenylglyoxal and (*p*-hydroxyphenyl)glyoxal may also react with certain protein sulfhydryls, especially highly reactive sulfhydryl groups, as well as with arginyl residues; the two reagents, although similar in structure, have different relative reactivities with sulfhydryl groups and with arginyl residues in the same protein and in different proteins (see below). This is in accord with the frequent observation that one of a series of related reagents may be more reactive with an active site residue in one enzyme whereas a different reagent may be more reactive with another enzyme. These differences depend on the environment of the active site residue and on the size, charge, and hydrophobicity of the reagent.

The recently introduced reagent (*p*-hydroxyphenyl)glyoxal (I, Chart I, λ_{max} at 335 nm) (Yamasaki et al., 1980) is very convenient because its product with arginine can be quantitated spectrophotometrically from its absorbance at 340 nm. The product contains 2 mol of (*p*-hydroxyphenyl)glyoxal/mol of arginine (Yamasaki et al., 1980) and presumably has structure II (Chart I), analogous to the structure that has been proposed for the 2:1 adduct formed by phenylglyoxal with arginine in the absence of borate (Takahashi, 1968; Werber et al., 1975).

Since (*p*-hydroxyphenyl)glyoxal (I) and its 2:1 adduct with arginine (II) have very similar extinction coefficients at 340 nm (Yamasaki et al., 1980), it seems likely that only one of the two *p*-hydroxyphenyl groups in II absorbs at 340 nm. The absorbing group is probably group A (structure II) in which the *p*-hydroxyphenyl group is conjugated to a carbonyl group; this conjugation is lost in group B. Structure III (Chart I) is a 1:1 adduct of (*p*-hydroxyphenyl)glyoxal and arginine with borate, analogous to the structure proposed for the 1:1 adduct of phenylglyoxal (Werber et al., 1981) or of butanedione (Riordan, 1973) formed in the presence of borate. Although (*p*-hydroxyphenyl)glyoxal has not previously been studied in borate buffer, we would expect (from the reasoning above) that the 1:1 adduct III, if formed, would not absorb at 340 nm. Indeed, our preliminary model experiments show that the absorbance at 340 nm of (*p*-hydroxyphenyl)glyoxal (20 μM) is lost upon reaction with *N*-(carbobenzoxy)arginine (up to 4 mM) in the presence of borate buffer.[3] Since (*p*-hydroxyphenyl)glyoxal does not modify arginyl residues in the α subunit but does modify cysteinyl residues, our results do not show whether structure II or structure III is formed when arginyl residues in a protein are modified by (*p*-hydroxyphenyl)glyoxal in the presence of borate. However, when proteins known to have reactive arginyl residues [e.g., the β_2 subunit of *E. coli* tryptophan synthase (Tanizawa & Miles, 1983) and *E. coli* alkaline phosphatase (Daemen & Riordan, 1974)] were modified with (*p*-hydroxyphenyl)glyoxal (2–20 mM) in the absence and presence of borate (100 mM) at pH 7.8, the modified and inactivated proteins were found to have equal absorbance at 340 nm under both conditions of modification.[3] These results suggest that the formation of the 2:1 adduct II (Chart I) is prevalent both in the presence and absence of borate ion, when the concentration of the (*p*-hydroxyphenyl)glyoxal (approximately 10 mM) is in large excess over the available arginines of a protein.

Phenylglyoxal forms a hemithioacetal with sulfhydryl groups (Schubert, 1936). An analogous structure for the (*p*-hydroxyphenyl)glyoxal adduct is IV (Chart I). Since this structure contains the *p*-hydroxyphenyl group conjugated to a carbonyl group, we would expect this adduct to absorb at 340 nm. The finding that the absorbance maximum at 335 nm of (*p*-hydroxyphenyl)glyoxal is shifted to a longer wavelength (343 nm) in the presence of excess *N*-acetylcysteine but does not change in amplitude[3] suggests that the thioacetal IV does absorb at 340 nm and has an extinction coefficient similar to that of (*p*-hydroxyphenyl)glyoxal and of the 2:1 adduct with arginine (II, Chart I). More extensive studies of the reactions of (*p*-hydroxyphenyl)glyoxal with model arginines and sulfhydryl compounds are needed to confirm these structures and to compare the rates of these reactions.

The structure of the product of reaction between phenylglyoxal or (*p*-hydroxyphenyl)glyoxal and the sulfhydryls of the α subunit has not been established. Although the formation of the hemithioacetal (IV) would be expected to be readily reversible, we observed no recovery of enzymatic activity nor diminution of the number of modifiable sulfhydryls after incubation of phenylglyoxal- or (*p*-hydroxyphenyl)glyoxal-modified α subunit with 0.2 M β-mercaptoethanol in 0.1 M sodium phosphate buffer, pH 7.0, and at 30 °C for 140 min. It is probable that the initially formed hemithioacetal undergoes a second, irreversible reaction with a suitably placed nucleophile on the protein.

The pseudo-first-order rate of inactivation of the α subunit by (*p*-hydroxyphenyl)glyoxal (10 mM), which appears to be entirely due to modification of sulfhydryl groups, is 0.035 min^{-1} at pH 7.8 and 30 °C (Figure 1). This is about 100 times faster than the pseudo-first-order rate of reaction of (*p*-hydroxyphenyl)glyoxal (10 mM) with 5-thio-2-nitrobenzoic acid, a model sulfhydryl compound.[3] thus, the reactivity of the essential sulfhydryl groups in the α subunit may be enhanced by their environment.

Since (*p*-hydroxyphenyl)glyoxal and phenylglyoxal appear selective in their reactivity with protein sulfhydryls (Table II), they may react only with sulfhydryls that are highly reactive or that have a certain particular environment. Neither reagent, for example, reacts with Cys-170 in the holo-β_2 subunit of *E. coli* tryptophan synthase, which is known to react with sulfhydryl-specific reagents such as *N*-ethylmaleimide and 5,5′-dithiobis(2-nitrobenzoic acid) but not with 2-nitro-5-(thiocyano)benzoic acid (Higgins & Miles, 1978; Miles & Higgins, 1980). (*p*-Hydroxyphenyl)glyoxal does react with one sulfhydryl in the apo-β_2 subunit. Since the apo form of the β_2 subunit is known to have a second exposed sulfhydryl (Cys-230) that reacts with 2-nitro-5-(thiocyano)benzoic acid, it appears that both (*p*-hydroxyphenyl)glyoxal and 2-nitro-5-(thiocyano)benzoic acid react selectively with this Cys-230 but not with Cys-170.

Modification of one or more of the three sulfhydryl groups of the α subunit by iodoacetate, *N*-ethylmaleimide, or 5,5′-dithiobis(2-nitrobenzoic acid) (Hardman & Yanofsky, 1965; Freedberg & Hardman, 1971b) or by phenylglyoxal or (*p*-hydroxyphenyl)glyoxal (this paper) results in inactivation. The number of sulfhydryl groups that react (1–3) with the different reagents and that appear to be essential for activity (1–2) probably depends on the characteristics of the reagent and on

the environment of each of the sulfhydryl groups. Modification by (*p*-hydroxyphenyl)glyoxal may result in a structural or conformational change, since the interaction of (*p*-hydroxyphenyl)glyoxal-modified α subunit with fresh β_2 subunit is impaired. The sulfhydryl groups are probably not directly involved in catalysis since none is conserved in the α sequence of the yeast tryptophan synthase (Zalkin & Yanofsky, 1982).

It is surprising that inactivation of the α subunit by (*p*-hydroxyphenyl)glyoxal results from modification of sulfhydryl groups and follows pseudo-first-order kinetics since inactivation of the α subunit by the sulfhydryl reagents methyl methanethiosulfonate,[3] iodoacetate (Hardman & Yanofsky, 1965), and *N*,*N*′-bis(maleimidomethyl) ether (Freedberg & Hardman, 1971a) does not follow first-order kinetics. The difference between the kinetics of inactivation by different sulfhydryl reagents could be explained if the more reactive sulfhydryl reagents (e.g., methyl methanethiosulfonate and iodoacetate) reacted with two or more sulfhydryl groups at different rates to yield some partially active species (Ray & Koshland, 1961; case IV) whereas the less reactive sulfhydryl reagent (*p*-hydroxyphenyl)glyoxal reacted more selectively.

Since inactivation of the α subunit by phenylglyoxal results from modification of three essential residues and follows pseudo-first-order kinetics, each essential residue is modified independently (Ray & Koshland, 1961; case I). Since phenylglyoxal binds loosely to the α subunit (K_I = 34 mM), it is not surprising that phenylglyoxal reacts less specifically than it does with an enzyme such as the β_2 subunit to which it binds tightly (K_I = 3.7 mM) and reacts with a single arginine (Tanizawa & Miles, 1983).

Although indole-3-glycerol phosphate and indole-3-propanol phosphate reduce the rate of inactivation of the α subunit by phenylglyoxal or (*p*-hydroxyphenyl)glyoxal, they do not reduce the final extent of sulfhydryl modification. Our finding that the estimated rate constant k_4 (see Table I and eq 2) is 1.7 times greater for (*p*-hydroxyphenyl)glyoxal than for phenylglyoxal and that (*p*-hydroxyphenyl)glyoxal is more reactive than phenylglyoxal with sulfhydryl groups (Table II) implies that the functional groups modified in the presence of protecting substrates or analogues are sulfhydryl groups.

Our conclusion that an essential arginyl residue is modified by phenylglyoxal and is protected by indole-3-glycerol phosphate is based on analyses of the kinetic data and of studies of total incorporation of phenyl[2-^{14}C]glyoxal and total sulfhydryl modification. The attempt to get a direct proof of an essential arginine by locating it in the peptide sequence is in progress.

Appendix

Kinetics of Partial Protection according to Equation 2. The dissociation constants are defined as

$$K_I = [E][I]/[EI] \quad (A1)$$

$$K_D = [E][S]/[ES] \quad (A2)$$

The total concentration of the enzyme $[E_0]$ is given by

$$[E_0] = [E_a] + [E_{ia}] \quad (A3)$$

where

$$[E_a] \text{ (active enzyme)} = [E] + [ES] + [EI] = [E](1 + [S]/K_D + [I]/K_I) \quad (A4)$$

and

$$[E_{ia}] \text{ (inactive enzyme)} = [EI^*] + [ESI'] \quad (A5)$$

Under the conditions of pseudo-first-order inactivation, i.e., $[E_0] \ll [I]$, and negligible turnover of the substrate, i.e., $[E_0] \ll [S]$, the rate of inactivation is given by

$$d[E_{ia}]/dt = -d[E_a]/dt = k_3[EI] + k_5[ES] = [E][k_3([I]/K_I) + k_5([S]/K_D)] \quad (A6)$$

where

$$k_5 = k_4[I] \quad (A7)$$

Rearranging eq A6 with eq A4

$$-d[E_a]/dt = k_{obsd}[E_a] \quad (A8)$$

where

$$k_{obsd} = \frac{k_3([I]/K_I) + k_5([S]/K_D)}{1 + [I]/K_I + [S]/K_D} \quad (A9)$$

Registry No. I, 24645-80-5; phenylglyoxal, 1074-12-0; L-arginine, 74-79-3; L-cysteine, 52-90-4; tryptophan synthase, 9014-52-2; indole-3-glycerol phosphate, 4220-97-7; indole-3-propanol phosphate, 40716-80-1.

References

Adachi, O., Kohn, L. D., & Miles, E. W. (1974) *J. Biol. Chem. 249*, 7756–7763.

Borders, C. L., Jr., & Riordan, J. F. (1975) *Biochemistry 14*, 4699–4704.

Crawford, I. P., Nichols, B. P., & Yanofsky, C. (1980) *J. Mol. Biol. 12*, 489–502.

Creighton, T. E., & Yanofsky, C. (1966) *J. Biol. Chem. 241*, 980–990.

Daemen, F. J. M., & Riordan, J. F. (1974) *Biochemistry 13*, 2865–2871.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Freedberg, W. B., & Hardman, J. K. (1971a) *J. Biol. Chem. 246*, 1439–1448.

Freedberg, W. B., & Hardman, J. K. (1971b) *J. Biol. Chem. 246*, 1449–1456.

Gavilanes, F., Peterson, D., & Schirch, L. V. (1982) *J. Biol. Chem. 257*, 11431–11436.

Gilbert, H. F., & O'Leary, M. H. (1975) *Biochemistry 14*, 5194–5199.

Guest, J. R., Drapeau, G. R., Carlton, B. C., & Yanofsky, C. (1967) *J. Biol. Chem. 242*, 5442–5446.

Hardman, J. K., & Yanofsky, C. (1965) *J. Biol. Chem. 240*, 725–732.

Harris, J. I., & Perham, R. N. (1965) *J. Mol. Biol. 13*, 876–884.

Hathaway, G. M., & Crawford, I. P. (1970) *Biochemistry 9*, 1801–1808.

Heyn, M. P., & Weischet, W. O. (1975) *Biochemistry 14*, 2962–2968.

Higgins, W., & Miles, E. W. (1978) *Biochem. Biophys. Res. Commun. 82*, 265–272.

Higgins, W., Fairwell, T., & Miles, E. W. (1979) *Biochemistry 18*, 4827–4835.

Kirschner, K., & Wiskocil, R. (1972) in *Protein-Protein Interactions* (Jaenicke, R., & Helmreich, E., Eds.) pp 245–268, Springer-Verlag, New York.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Kohlbrenner, W. E., & Cross, R. L. (1978) *J. Biol. Chem. 253*, 7609–7611.

Kuno, S., Toraya, T., & Fukui, S. (1980) *Arch. Biochem. Biophys. 205*, 240–245.

Li, S.-L., & Yanofsky, C. (1973) *J. Biol. Chem. 248*, 1837–1843.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, E. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mäkinen, K. K., Mäkinen, P.-L., Wilkes, S. H., Bayliss, M. E., & Prescott, J. M. (1982) *J. Biol. Chem. 257*, 1765–1772.
Malkinson, A. M., & Hardman, J. K. (1969) *Biochemistry 8*, 2769–2776.
Miles, E. W. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 127–186.
Miles, E. W., & Moriguchi, M. (1977) *J. Biol. Chem. 252*, 6594–6599.
Miles, E. W., & Higgins, W. (1980) *Biochem. Biophys. Res. Commun. 93*, 1152–1159.
Miles, E. W., & Eun, H.-M. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 1346 (Abstr. 756).
Patthy, L., & Thész, J. (1980) *Eur. J. Biochem. 105*, 387–393.
Peters, R. G., Jones, W. C., & Cromartie, T. H. (1981) *Biochemistry 20*, 2564–2571.
Platt, T. (1978) in *The Operon* (Miller, J. H., & Regnikoff, W. S., Eds.) pp 263–302, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Ray, W. J., Jr., & Koshland, D. E., Jr. (1961) *J. Biol. Chem. 236*, 1973–1979.
Riddles, P. W., Blakeley, R. L., & Zerner, G. (1983) *Methods Enzymol. 91*, 49–60.
Riordan, J. F. (1973) *Biochemistry 12*, 3915–3923.
Riordan, J. F., McElvany, K. D., & Borders, G. L., Jr. (1977) *Science (Washington, D.C.) 195*, 884–886.
Schubert, M. P. (1936) *J. Biol. Chem. 114*, 341–350.
Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.
Takahashi, K. (1977) *J. Biochem. (Tokyo) 81*, 395–402.
Tanizawa, K., & Miles, E. W. (1983) *Biochemistry 22*, 3594–3603.
Tschopp, J., & Kirschner, K. (1980) *Biochemistry 19*, 4514–4521.
Tsou, C.-L. (1962) *Sci. Sin. (Engl. Ed.) 11*, 1535–1558.
Tuszynski, G. P., Knight, L., Piperno, J. R., & Walsh, P. N. (1980) *Anal. Biochem. 106*, 118–122.
Vandenbunder, B., Dreyfus, M., Bertrand, O., Dognin, M. J., Sibilli, L., & Buc, H. (1981) *Biochemistry 20*, 2354–2360.
Weischet, W. O., & Kirschner, K. (1976) *Eur. J. Biochem. 65*, 365–373.
Weng, L., Heinrikson, R. L., & Westley, J. (1978) *J. Biol. Chem. 253*, 8109–8119.
Werber, M. M., Moldovan, M., & Sokolovsky, M. (1975) *Eur. J. Biochem. 53*, 207–216.
Wilson, D. A., & Crawford, I. P. (1965) *J. Biol. Chem. 240*, 4801–4808.
Yamasaki, R. B., Vega, A., & Feeney, R. E. (1980) *Anal. Biochem. 109*, 32–40.
Yamasaki, R. B., Shimer, D. A., & Feeney, R. E. (1981) *Anal. Biochem. 111*, 220–226.
Yanofsky, C., & Crawford, I. P. (1972) *Enzymes, 3rd Ed. 7*, 1–31.
Yanofsky, C., Platt, T., Crawford, I. P., Nichols, B. P., Christie, G. E., Horowitz, H., Van Cleemput, M., & Wu, A. M. (1981) *Nucleic Acids Res. 9*, 6647–6668.
Zalkin, H., & Yanofsky, C. (1982) *J. Biol. Chem. 257*, 1491–1500.

Isolation of a Protein Fraction That Binds Preferentially to Chicken Middle Repetitive DNA†

Michael Sanzo,‡ Bryn Stevens, Ming-Jer Tsai, and Bert W. O'Malley*

ABSTRACT: We have fractionated oviduct tissue extracts by using a combination of ion-exchange and DNA–Sephadex chromatography. By comparing the electrophoretic patterns of proteins eluted from competing specific and nonspecific DNA columns, we isolated a fraction which bound with specificity to columns containing the chicken middle repetitive sequence "CR1". This fraction showed a clear preference for binding to separate, cloned CR1 fragments derived from either the 5′ or the 3′ transition region of the ovalbumin gene domain when examined by using nitrocellulose filter binding assays. To localize the protein binding site, a CR1 clone was digested with various restriction enzymes, and the resulting fragments were examined for preferential protein binding. Results suggest that the binding site lies within a 39-nucleotide sequence which is highly conserved among different CR1 elements. This finding represents the first isolation of a protein which demonstrates a preference for binding to a middle repetitive sequence and suggests that this interaction may have a biological role. The DNA column competition adsorption method should have general application to the isolation of other gene-regulating proteins possessing DNA sequence preference.

The DNA of a large number of species has been shown to contain certain nucleotide sequences which are repeated many times and whose copies are interspersed among structural genes (Davidson et al., 1975, 1977; Schmid & Deininger, 1975). In chicken cells, a family of short (250 nucleotides) repetitive elements, designated "CR1", is present in about 7000 copies per genome (Stumph et al., 1981). CR1 sequences have been shown to contain regions homologous with Alu repetitive elements from the human and with B1 repetitive elements from the mouse and to contain short direct repeats, a characteristic common to many transposable elements (Stumph et al., 1981). Of particular interest is the positioning of CR1 elements in relation to the ovalbumin gene. In oviduct tissue, the ovalbumin gene is highly transcribed and is located within a region

† From the Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030. *Received May 21, 1984.* This work was supported by grants from the National Institutes of Health (HD-08188 and HD-07495).
‡ Present address: Division of Laboratory Medicine, Washington University School of Medicine, St. Louis, MO 63110.

0006-2960/84/0423-6491$01.50/0