A Study of the Quenching of the Intrinsic Fluorescence of Succinyl-CoA Synthetase from *Escherichia coli* by Acrylamide, Iodide, and Coenzyme A†

A. R. S. Prasad, Jonathan S. Nishimura, and Paul M. Horowitz*

ABSTRACT: *Escherichia coli* succinyl-CoA synthetase (SCS) contains three tryptophan residues per mole of $\alpha\beta$ dimer, and all of them are on the β subunit. SCS shows an emission maximum at 335 nm which is shifted to 350 nm upon denaturation by urea or guanidine hydrochloride. Acrylamide is able to quench the tryptophan fluorescence in SCS by static and dynamic mechanisms. Substrates give protection against quenching by acrylamide. Binding of ATP to the α subunit which has no tryptophans gives as large an effect on the quenching by acrylamide as the binding of coenzyme A (CoA) to the β subunit. Addition of CoA eliminates the curvature observed in Stern–Volmer plots for acrylamide quenching obtained by lifetime measurements. Potassium iodide does not quench the SCS fluorescence in the presence of CoA. These results suggest that there are heterogeneously emitting tryptophan residues in SCS that are located at the $\alpha\beta$ subunit contact region close to the CoA binding site. Hence, the tryptophan residues can act as intrinsic reporters of events taking place at the active site of this enzyme. Further, the present results support models for SCS that put the active site at the $\alpha\beta$ subunit contact region.

Succinyl-CoA synthetase (SCS)[1] (EC 6.2.1.5) of *Escherichia coli* catalyzes the following reaction:

$$\text{succinate} + \text{ATP} + \text{CoA} \leftrightarrow \text{succinyl-CoA} + \text{ADP} + P_i$$

The enzyme has an $\alpha_2\beta_2$ structure; i.e., it is a dimer of $\alpha\beta$ dimers (Bridger, 1971). The two active sites appear to be located at the points of contact between α- and β-polypeptide chains (Bridger, 1981). The enzyme mechanism involves a phosphoenzyme intermediate, and the site of this phosphorylation is a histidine residue of the α subunit (Bridger, 1971). An important sulfhydryl group also appears to be close to the CoA binding site on the β subunit (Collier & Nishimura, 1978). Phosphorylation of the isolated α subunit by ATP is significantly stimulated by addition of the β subunit (Pearson & Bridger, 1975). Moreover, partial reactions catalyzed by the enzyme are greatly stimulated by other substrates or analogues (Bridger et al., 1968; Grinnell & Nishimura, 1969a,b; Hildebrand & Spector, 1969). An interpretation of these observations is that binding of all substrates results in the proper alignment of amino acid side chains such that catalysis can occur. Implicit in this is the concept that binding of substrates brings about conformational changes in the region of the active site. Recently, we have been able to show through fluorescence measurements on dansyl-labeled enzyme that the regions of the molecules bearing a dansyl group, presumably close to the active site, showed greatly increased flexibility when CoA was present (Prasad et al., 1982). Because both subunits contained the dansyl label, it was not possible to determine where, α subunit, β subunit, or both, the flexible regions lay. Since the enzyme is known to contain tryptophan and a significant amount of tyrosine (Leitzmann et al., 1970; Bridger, 1974), we decided to examine the intrinsic fluorescence of the enzyme and to determine if binding of substrates had any effect on this fluorescence.

In this paper, we report the results of a study of the subunit location of the tryptophan residues and the effects of various quenchers and substrates on the intrinsic fluorescence of this enzyme.

Materials and Methods

All chemicals used were reagent grade. CoA (lithium salt) was obtained from P-L Biochemicals. *N*-Acetyltryptophanamide was from Sigma. Pronase and chymotrypsin were from Worthington. Electrophoresis-grade acrylamide was a product of Bio-Rad.

Succinyl-CoA synthetase was purified and assayed according to the previously published procedures (Grinnell & Nishimura, 1969a; Bowman & Nishimura, 1975). Subunits of succinyl-CoA synthetase were isolated on a Sephadex G-150 column developed in a buffer containing 6 M urea, 5% acetic acid, 0.1 mM EDTA, and 0.5 mM dithiothreitol by the method of Pearson & Bridger (1975).

Tryptophan content of the native enzyme and isolated α and β subunits was determined according to the procedure of Sasaki et al. (1975) which involves fluorescence measurement after digestion of the protein by chymotrypsin and Pronase. Lactate dehydrogenase and bovine serum albumin were used as reference proteins to check the values obtained.

Fluorescence quenching studies were done by using either an Aminco Bowman spectrofluorometer or a Perkin-Elmer MPF 44a spectrofluorometer. A solution of *N*-acetyltryptophanamide giving a fluorescence intensity comparable to the sample was used to check for the instrumental variation. Fluorescence lifetime measurements were made on an SLM Model 4800 spectrophotofluorometer (SLM Instruments, Urbana, IL) in the phase-modulation cross-correlation configuration by using a modulating frequency of 18 MHz. The lifetimes reported were determined from the phase measurements. All measurements were done at 20 °C.

The fluorescence quenching data were analyzed by using a Stern–Volmer-type equation which relates the decrease in fluorescence to the concentration of quencher as follows:

$$F_0/F = (1 + K_{SV}[Q])e^{V[Q]} \quad (1)$$

† From the Department of Biochemistry, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284. *Received December 20, 1982.* Supported by Grants AQ-458 (to J.S.N.) and AQ-723 (to P.M.H.) from the Robert A. Welch Foundation and Grants GM-17534 (to J.S.N.) and GM-25177 (to P.M.H.) from the U.S. Public Health Service.

[1] Abbreviations: CoA, coenzyme A; SCS, succinyl-CoA synthetase; dansyl, 8-(dimethylamino)-1-naphthalenesulfonyl; EDTA, ethylenediaminetetraacetic acid.

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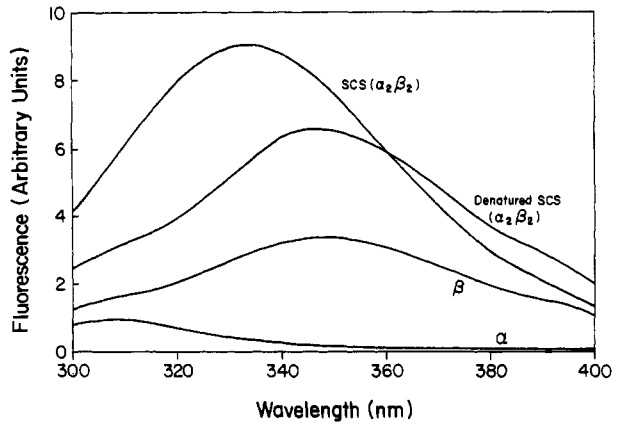

FIGURE 1: Fluorescence emission spectra of *E. coli* succinyl-CoA synthetase and its subunits. Excitation was at 280 nm. Spectra are corrected for the buffers. (a) Spectrum of native $\alpha_2\beta_2$ tetramer in 50 mM phosphate, pH 7.0. (b) Spectrum of denatured ($\alpha_2\beta_2$) enzyme in acid–urea. (c) Spectrum of isolated β subunit in acid–urea. (d) Spectrum of isolated α subunit in acid–urea.

where F and F_0 are the fluorescence intensities in the presence and absence of a quencher, respectively, [Q] is the concentration of quencher, V is the static quenching constant, and K_{SV} is the Stern–Volmer constant for the quenching process and is equal to $k_q\tau_0$ where k_q is the bimolecular rate constant for the quenching process and τ_0 is the fluorescence lifetime in the absence of quencher. A plot of F_0/F vs. [Q] is used to describe the process.

If there is only dynamic quenching, F_0/F and τ_0/τ become proportional and eq 1 becomes

$$F_0/F = \tau_0/\tau = 1 + K_{SV}[Q] \quad (2)$$

where τ is the fluorescence lifetime in the presence of quencher. Hence, a plot of F_0/F or τ_0/τ against [Q] should be linear for a homogeneous population of emitting fluorophores subject only to dynamic quenching (Eftink & Ghiron, 1981).

A modified form of the Stern–Volmer equation (eq 2) was used to analyze the quenching of heterogeneously emitting fluorophores (Lehrer, 1971):

$$\frac{F_0}{F_0 - F} = \frac{1}{[Q]f_a K_{SV}} + \frac{1}{f_a} \quad (3)$$

where f_a = the maximum fraction of the protein fluorescence accessible to quencher. A plot of $F_0/(F_0 - F)$ vs. $1/[Q]$ will yield a straight line having a slope of $1/(f_a K_{SV})$ and an intercept of $1/f_a$.

A correction for the CoA absorption in fluorescence quenching studies was done according to McClure & Edelman (1967). The total absorption of the sample never exceeded 0.14 at 295 nm.

Results and Discussion

When excited at 280 nm, *Escherichia coli* succinyl-CoA synthetase exhibited an emission spectrum as shown in Figure 1. The emission maximum (335 nm) and the spectral band width were not greatly altered by varying the excitation wavelength from 280 to 295 nm. Excitation at 295 nm, however, led to disappearance of the slight shoulder at 308 nm, suggesting a small contribution of tyrosine residues to the fluorescence spectrum of the enzyme. Denaturation in acid–urea (Materials and Methods) or 6.7 M guanidine hydrochloride, pH 7.5, shifted the emission maximum to 350 nm, which is characteristic of fully exposed tryptophan residues (Figure 1). Comparison of fluorescence emission spectra of isolated α and β subunits shows that there is very little fluorescence from the α subunit, suggesting that all the tryptophan residues of SCS which fluoresce under these conditions are located on the β subunit (Figure 1). Since this experiment was performed under denaturing conditions, it seemed likely that all the tryptophans in SCS were on the β subunit. As there was no available information about the distribution of the tryptophan residues between α and β subunits, we made a quantitative estimation of tryptophan residues in the native enzyme and isolated α and β subunits by using the method of Sasaki et al. (1975). The results are shown in Table I. Native succinyl-CoA synthetase has three tryptophan residues per mole of the dimer. Earlier reported values of four tryptophans per mole of dimer were based on the UV absorption of the enzyme, but possible local interactions in the protein were not specifically accounted for (Bridger, 1974). The results here show that the β subunit contains all of the tryptophan residues and give a value of three residues per mole of the subunit. These results are within the limits of experimental error as shown by the values obtained by using the present method for other proteins whose tryptophan contents have been well characterized.

Table I: Tryptophan Content of Succinyl-CoA Synthetase and Its Subunits[a]

protein	tryptophan (no. of residues/mol of protein)
succinyl-CoA synthetase ($\alpha\beta$ dimer)	2.91
α subunit	0.13
β subunit	3.05
lactate dehydrogenase	22.46[b]
bovine serum albumin	2.4[c]

[a] Two nanomoles of each protein was digested with a mixture of chymotrypsin and Pronase, and the tryptophan content was determined according to the method of Sasaki et al. (1975). [b] Literature value of 20–23 reported in Pesce et al. (1964) and Goodwin & Morton (1946). [c] Literature value of 2 reported in King & Spencer (1970).

Acrylamide is known to be capable of quenching the tryptophan fluorescence in proteins by either a static mechanism involving complex formation or a dynamic quenching mechanism involving collisions with acrylamide during the lifetime of the tryptophan excited state, or both. The two mechanisms can be differentiated experimentally by comparing changes in the fluorescence intensity with changes in the fluorescence lifetime as a function of the acrylamide concentration. As shown in Figure 2, acrylamide was able to quench the fluorescence of succinyl-CoA synthetase. The Stern–Volmer plot obtained by fluorescence intensity measurements shows an upward curvature which is often indicative of the presence of static quenching. The linear portion of the plot at low acrylamide concentration [(●) in Figure 2A] gave a Stern–Volmer constant (K_{SV}) of 6.5 M^{-1} for the native enzyme. The quenching did not appear to result from gross changes in protein conformation induced by acrylamide for the following reasons: (a) the emission spectrum retained the characteristic emission maximum of the native enzyme even in the presence of acrylamide; (b) the enzyme was fully active in the presence of acrylamide up to 1 M; and (c) actual denaturation of the enzyme with 6.7 M guanidine hydrochloride led to increased quenching and a greater positive deviation of the Stern–Volmer plot and gave a Stern–Volmer constant of 10.2 M^{-1} (Figure 2A) in addition to the previously noted changes in the intrinsic fluorescence.

Experiments were conducted to study whether ligand interactions with the enzyme could induce changes in the ac-

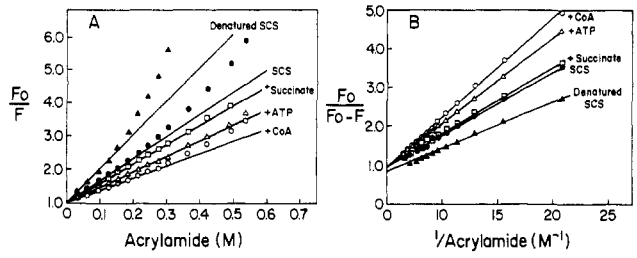

FIGURE 2: Effect of substrates on acrylamide quenching of tryptophan fluorescence in *E. coli* succinyl-CoA synthetase. SCS at a concentration of 0.1 mg/mL was preincubated with 1–5 mM levels of various substrates for 30 min, after which acrylamide was added and fluorescence quenching measured. Denaturation of SCS was in 6.7 M guanidine hydrochloride, pH 7.5. Excitation was at 295 nm. Emission was monitored at 335 nm for native enzyme and at 350 nm for denatured enzyme. Figure 2A shows the Stern–Volmer plot, and Figure 2B shows the modified Stern–Volmer plot.

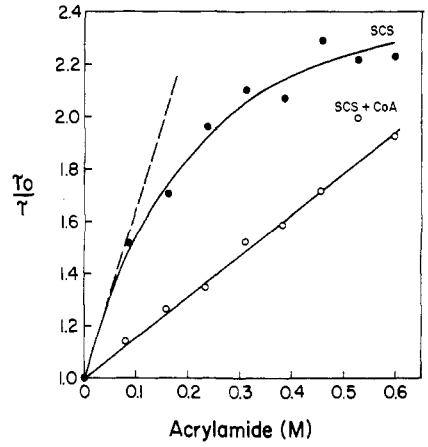

FIGURE 3: Stern–Volmer plot for the acrylamide quenching of succinyl-CoA synthetase fluorescence in the presence and absence of CoA. SCS at a concentration of 0.1 mg/mL was preincubated with 1 mM CoA in 100 mM phosphate buffer, pH 7.5. Lifetime measurements were made with the excitation at 295 nm as described under Materials and Methods.

cessibility of tryptophan residues, as reflected in changes in fluorescence quenching patterns. For example, individual additions of the substrates succinate, ATP, and CoA to succinyl-CoA synthetase each decreased the effectiveness of quenching by acrylamide, as shown in Figure 2A. The largest effect was obtained with CoA. The Stern–Volmer constant (K_{SV}) decreased from 6.5 to 4 M^{-1} in the presence of 1 mM CoA. A similar decrease in the quenching constant has been observed on the binding of a competitive inhibitor to β-trypsin (Ramachandran, 1973) and on the binding of *N*-acetylglucosamine to lysozyme (Eftink & Ghiron, 1976). ATP which binds at the α subunit also brings about a decrease in K_{SV}. It is interesting that the binding of ATP to the subunit with no tryptophans gives almost as large an effect on the quenching by acrylamide as CoA gives. Figure 2B shows the modified Stern–Volmer plot obtained for the quenching of tryptophan fluorescence in SCS. As all the lines give the same intercept, it follows that all the tryptophan fluorescence in SCS is totally accessible at sufficiently high concentration but the effectiveness of acrylamide, as reflected in the K_{SV} values, varies according to the substrates that are present. $K_{SV} = k_q\tau_0$ where τ_0 is the lifetime in the absence of quencher. As K_{SV} is 6.5 M^{-1} and τ_0 is 3.6 ns, k_q can be calculated as 1.8×10^9 M^{-1} s^{-1}. A value of $(3–5) \times 10^9$ M^{-1} s^{-1} has been reported for fully exposed tryptophan residues (Eftink & Ghiron, 1975).

Figure 3 shows the Stern–Volmer plot obtained by lifetime measurements in the presence and absence of CoA. In the absence of CoA, the plot is concave toward the abscissa, which is characteristic of heterogeneously emitting tryptophan residues. This nonlinearity was removed in the presence of 1 mM CoA. This curvature in the Stern–Volmer plot is a result of certain tryptophans being quenched preferentially over others in a protein. The portion of the Stern–Volmer plot at low acrylamide concentrations corresponds largely to the quenching of more easily accessible residues. At higher concentrations of quencher, the tryptophans which are difficult to quench become more obvious. Hence, it appears that there are at least two kinds of tryptophan residues in SCS which are quenched by a collisional mechanism. Binding of CoA seems to selectively quench the tryptophan residues with the lower quenching constant and remove the nonlinearity. In addition, CoA also results in a decreased apparent Stern–Volmer constant (K_{SV}) as also shown in Figure 2A.

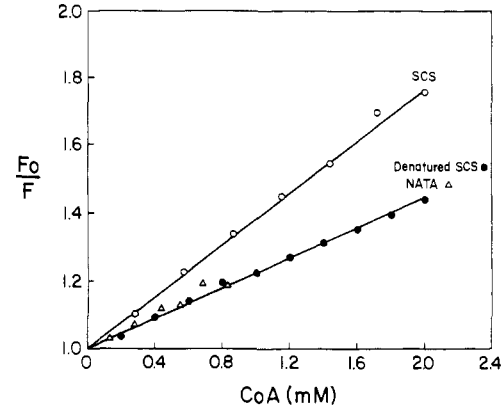

FIGURE 4: Stern–Volmer plot for the CoA quenching of tryptophan fluorescence in *E. coli* succinyl-CoA synthetase. SCS was at 0.1 mg/mL. Native enzyme and *N*-acetyltryptophanamide (NATA) were in 100 mM phosphate, pH 7.5. Denatured enzyme was in 6.7 M guanidine hydrochloride, pH 7.5. Excitation was at 295 nm. Emission was measured at 335 nm for native SCS and at 350 nm for denatured SCS and NATA.

In order to understand about the effects observed with CoA addition, we constructed a Stern–Volmer plot for the quenching of SCS fluorescence by CoA itself. The results are shown in Figure 4. In these plots, the fluorescence intensities have been corrected for the absorption due to CoA at 295 nm. CoA acts as a very good quencher of tryptophan fluorescence in SCS and gives a value for K_{SV} of 0.36 mM^{-1}. Denaturation of SCS with 6.7 M guanidine hydrochloride brings about decreased quenching and gives a value of 0.2 mM^{-1} for the Stern–Volmer constant. This latter value is very similar to that obtained for the CoA quenching of *N*-acetyltryptophanamide. Destruction of the CoA binding site by denaturation of the enzyme would be expected to result in a decreased quenching constant as observed. These results suggest that there are tryptophan residues at or near the CoA binding site and emphasize the necessity for the specific binding of CoA. Our earlier studies have shown that the binding of CoA does not bring about any large conformational changes in SCS (Prasad et al., 1982).

In order to understand more about the environment of tryptophan residues in succinyl-CoA synthetase, we performed quenching studies with the anionic quencher, iodide ion. Iodide ion, being negatively charged and hydrated, is likely to quench only surface tryptophanyl residues, and its behavior should also depend on the neighboring charged groups (Lehrer, 1971). The quenching profiles obtained in the presence and absence of CoA are shown in Figure 5A. The Stern–Volmer plot at constant ionic strength gave a K_{SV} for iodide of 1.39 M^{-1}. In the presence of 1 mM CoA, there was no quenching of tryp-

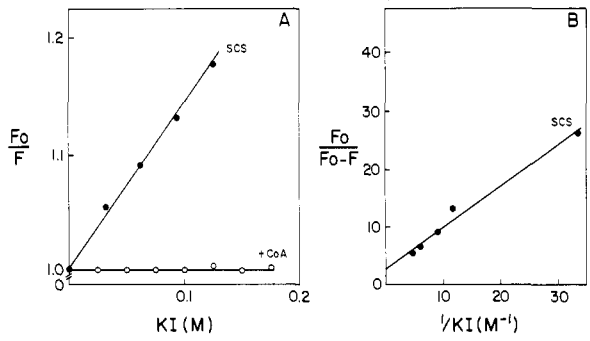

FIGURE 5: Stern–Volmer plot for the potassium iodide quenching of tryptophan fluorescence in *E. coli* succinyl-CoA synthetase in the absence or presence of 1 mM CoA. The ionic strength was maintained at a constant value of 0.2 M with the addition of potassium chloride. Protein concentration was 0.1 mg/mL in 100 mM phosphate, pH 7.5. Excitation was at 295 nm. Emission was at 335 nm. (A) Stern–Volmer plot; (B) modified Stern–Volmer plot.

tophanyl fluorescence in SCS by iodide. This suggested that the tryptophans quenched by iodide are also the ones that are quenched by CoA. The modified Stern–Volmer plot in Figure 5B, for the iodide quenching of SCS fluorescence, gives a value of 0.45 for f_a. To the extent that each tryptophan contributes equally to the protein fluorescence in the native enzyme, this also suggests that iodide is able to quench the fluorescence of only one tryptophan residue which is close to the CoA binding site.

This last conclusion must remain speculative since, due to the variability of the quantum yields of individual tryptophan residues, it is not formally possible to equate fractional accessibilities to the number of residues involved (Eftink & Ghiron, 1981).

It therefore appears on the basis of these quenching studies that the tryptophan residues in SCS can be divided into three categories as follows: (i) those residues that are quenched by a static mechanism; (ii) those residues that are quenched by a collisional mechanism but not protected by CoA; and (iii) those residues that are quenched by a collisional mechanism and protected by CoA. As there are three tryptophan residues per mole of the $\alpha\beta$ dimer, it is of interest to know if each one is quenched by one of the three mechanisms or if each is partially quenched by more than one mechanism. At present, we do not have any data to ascertain this.

Cross-linking studies using the sulfhydryl cross-linker *o*-phenylenedimaleimide have shown that a sulfhydryl group at or near the CoA binding site on the β subunit is quite close to a sulfhydryl group on the α subunit (Collier & Nishimura, 1978). The present observation that binding of ATP at the α subunit caused a decrease in the fluorescence quenching by acrylamide is consistent with the suggestion that the active site of the enzyme very likely is located at the interface between α and β subunits (Bridger, 1981). All these results clearly suggest the presence of at least one tryptophan residue at the $\alpha\beta$ subunit interface on the β subunit, close to the CoA binding site. Further, the polar environment of this residue is suggested by iodide quenching and its protection by CoA binding. The nature of the fluorescence spectra of native SCS together with the quenching patterns observed here would suggest that the tryptophan(s) whose fluorescence is affected by CoA binding is (are) class II residues in the Burstein classification (Burstein et al., 1973): surface residues with somewhat restricted access to the solvent. This situation would be realized if these residues were at the intersubunit contact region, and this suggestion is consistent with the other information presented here. Vogel & Bridger (1982) have shown by ^{31}P NMR studies that the binding of CoA causes a downfield shift and broadening of the phosphohistidyl resonance in SCS. This has been attributed to changes in SCS structure as a result of CoA binding. Our recent investigation using the dansyl derivative of SCS has shown that there are flexible segments in this protein and their flexibility is altered by the binding of CoA (Prasad et al., 1982). One of the limitations of this approach was the possibility of an alteration in the protein structure as a result of dansylation. The results here are clearly consistent with models for SCS that place the active site at the $\alpha\beta$ interface and provide an intrinsic reporter of events at this region of the intact protein.

Registry No. SCS, 9080-33-5; CoA, 85-61-0; iodide, 20461-54-5; acrylamide, 79-06-1; tryptophan, 73-22-3.

References

Bowman, C. M., & Nishimura, J. S. (1975) *J. Biol. Chem. 250*, 5609–5613.

Bridger, W. A. (1971) *Biochem. Biophys. Res. Commun. 42*, 948–954.

Bridger, W. A. (1974) *Enzymes, 3rd Ed. 10*, 581–606.

Bridger, W. A. (1981) *Can. J. Biochem. 59*, 1–8.

Bridger, W. A., Millen, W. A., & Boyer, P. D. (1968) *Biochemistry 7*, 3608–3616.

Burstein, E. A., Vedekina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.

Collier, G. E., & Nishimura, J. S. (1978) *J. Biol. Chem. 253*, 4938–4943.

Eftink, M. R., & Ghiron, C. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3290–3294.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15*, 672–680.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.

Goodwin, T. W., & Morton, R. A. (1946) *Biochem. J. 40*, 628–632.

Grinnell, F. L., & Nishimura, J. S. (1969a) *Biochemistry 8*, 562–568.

Grinnell, F. L., & Nishimura, J. S. (1969b) *Biochemistry 8*, 568–574.

Hildebrand, J. G., & Spector, L. B. (1969) *J. Biol. Chem. 244*, 2606–2613.

King, T. P., & Spencer, M. (1970) *J. Biol. Chem. 245*, 6134–6148.

Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3263.

Leitzmann, C., Wu, J., & Boyer, P. D. (1970) *Biochemistry 9*, 2338–2346.

McClure, W. O., & Edelman, G. M. (1967) *Biochemistry 6*, 559–566.

Pearson, P. H., & Bridger, W. A. (1975) *J. Biol. Chem. 250*, 8524–8529.

Pesce, A., McKay, R. H., Stolzenbach, F., Cahn, R. D., & Kaplan, N. O. (1964) *J. Biol. Chem. 239*, 1753–1761.

Prasad, A. R. S., Nishimura, J. S., & Horowitz, P. M. (1982) *Biochemistry 21*, 5142–5147.

Ramachandran, N. (1973) Ph.D. Thesis, University of Missouri, Columbia, MO.

Sasaki, T., Abrams, B., & Horecker, B. L. (1975) *Anal. Biochem. 65*, 396–404.

Vogel, H. J., & Bridger, W. A. (1982) *J. Biol. Chem. 257*, 4838–4842.