Hartshorne, D. J., Gőrecka, A., & Aksoy, M. O. (1977) in *Excitation-Contraction-Coupling in Smooth Muscle* (Casteels, R., Godfraind, T., & Rüegg, J. C., Eds.) pp 377–384, Elsevier/North-Holland Biomedical Press, Amsterdam.
Itzhaki, R. F., & Gill, D. M. (1964) *Anal. Biochem. 9*, 401–410.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Lambin, P. (1978) *Anal. Biochem. 85*, 114–125.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
Marston, S. B., Trevett, R. M., & Walters, M. (1980) *Biochem. J. 185*, 355–365.
Mikawa, T., Nonomura, Y., Hirata, M., Ebashi, S., & Kakiuchi, S. (1978) *J. Biochem. (Tokyo) 84*, 1633–1636.
Mrwa, U., & Hartshorne, D. J. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1564–1568.
Murray, K. J., & England, P. J. (1980) *Biochem. J. 192*, 967–970.
Nonomura, Y., & Ebashi, S. (1980) *Biomed. Res. 1*, 1–14.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31–38.
Persechini, A., Mrwa, U., & Hartshorne, D. J. (1981) *Biochem. Biophys. Res. Commun. 98*, 800–805.
Peters, K. A., Demaille, J. G., & Fischer, E. H. (1977) *Biochemistry 16*, 5691–5697.
Spector, T. (1978) *Anal. Biochem. 86*, 142–146.
Walsh, M. P., Cavadore, J.-C., Vallet, B., & Demaille, J. G. (1980) *Can. J. Biochem. 58*, 299–308.
Walters, M., & Marston, S. B. (1981) *Biochem. J. 197*, 127–139.

Fluorescently Labeled Myosin Subfragment 1: Identification of the Kinetic Step Associated with the Adenosine 5′-Triphosphate Induced Fluorescence Decrease†

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ABSTRACT: Marsh and Lowey [Marsh, D. J., & Lowey, S. (1980) *Biochemistry 19*, 774–784] introduced a fluorescence label, 5-(iodoacetamido)fluorescein (IAF), into the alkali 1 light chain of myosin subfragment 1 (S-1) without perturbing the K^+-, Ca^{2+}-, or actin-activated ATPase activities of the labeled S-1. They also found that the addition of MgATP to the labeled S-1 led to a 6–10% decrease in the fluorescence of the bound IAF label. In the present study, we investigated the pre-steady-state kinetics of the fluorescence decrease of the IAF label caused by the binding of MgATP to the labeled S-1. Our results show that most of the decrease in IAF fluorescence is caused by the ATP-hydrolysis step. This implies that a conformational change in the S-1 molecule associated with ATP hydrolysis has led to a change in the microenvironment of the IAF fluorophore bound to the alkali 1 light chain.

The interaction of ATP with skeletal muscle myosin is accompanied by a significant increase in tryptophan fluorescence (Werber et al., 1972). Recently it has become apparent that most of this fluorescence enhancement is due to the ATP-hydrolysis step rather than the binding of ATP (Johnson & Taylor, 1978; Chock et al., 1979). The ATP hydrolysis step follows the binding of ATP as shown in the kinetic model for the myosin ATPase activity:

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M\cdot ATP \overset{K_2}{\rightleftharpoons} M^*\cdot ATP \overset{K_3}{\rightleftharpoons} M^{**}\cdot ADP\cdot P_i \overset{K_4}{\rightleftharpoons} M + ADP + P_i$$

where M is myosin, P_i is phosphate, and the stars indicate conformational states of myosin corresponding to different degrees of tryptophan fluorescence enhancement. The rate-limiting step in this model is the very slow conformational change which allows release of P_i (step 4), while the first-order step which causes the fluorescence enhancement (step 3) occurs quite rapidly and is responsible for the initial phosphate burst (Bagshaw & Trentham, 1974; Bagshaw et al., 1974).

Recently, Marsh & Lowey (1980) have been able to introduce a fluorescent label into the alkali 1 light chain of myosin subfragement 1 (S-1) without perturbing the K^+-, Ca^{2+}-, or actin-activated ATPase activities of the enzyme. These authors also found that the addition of MgATP to the labeled subfragment 1 causes a 6–10% decrease in the fluorescence of the label.

In the present study we investigated the pre-steady-state kinetics of the extrinsic fluorescence decrease which occurs when ATP interacts with subfragment 1 labeled on the alkali 1 light chain with 5-(iodoacetamido)fluorescein (IAF). In particular, we wished to establish which step in the kinetic cycle was responsible for the decrease in IAF fluorescence. Our results show that, as with the increase in intrinsic tryptophan fluorescence, much of the decrease in IAF fluorescence is caused by the ATP-hydrolysis step. It would appear that the conformational change associated with ATP hydrolysis has an effect on the microenvironment of the IAF fluorophore bound to the alkali 1 light chain.

Materials and Methods

Protein Preparations. Actin was prepared and its concentration determined as previously described (Fraser et al., 1975). The preparation and characterization of myosin subfragment

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1 containing an alkali 1 light chain labeled with IAF at its single cysteine residue have been described in detail previously (Marsh & Lowey, 1980). The purified, fluorescently labeled subfragment 1 obtained by the last step of this procedure (ion-exchange chromatography) was generally in dilute solution (approximately 0.1–0.3 mg/mL). Before use in the kinetic studies, it was concentrated to 1–2 mg/mL by vacuum dialysis or by immersion of a dialysis sac containing the protein in Sephadex G-200. During concentration the labeled subfragment 1 was kept in 10 mM imidazole, 1 mM dithiothreitol (DTT), and 10 mg/mL sucrose, pH 7.0, at 4 °C. It was then frozen in a dry ice–propanol mixture and kept on dry ice until being thawed immediately before use. Each preparation was used within 2 days of being frozen. One preparation was not frozen but was kept for 0.5 day before use in a dialysis sac bathed in the above buffer at 0 °C.

The high concentration of sucrose was employed in order to minimize loss of enzymatic activity following earlier indications that the ATPase activity of the labeled subfragment 1 was less stable than that of unlabeled subfragment 1. Subsequent observations confirmed this suspicion, although it was found that, for the one preparation of labeled subfragment 1 examined for loss of activity, full ATPase activity was retained for 3 days after the last stage of its preparation when stored on ice in the presence of sucrose. However, the relative instability of the labeled proteins, and the limited quantities available, restricted the measurements to fewer conditions than would have been the case with unlabeled subfragment 1.

The steady-state K^+- and Ca^{2+}-ATPase activities and the decrease in IAF fluorescence upon the addition of ATP were examined for each preparation of labeled subfragment 1 and were found to be similar to the values previously reported (Marsh & Lowey, 1980). The ATPase measurements were performed by using a Radiometer SBR2 titrigraph thermostated at 25.0 ± 0.1 °C, and the fluorescence measurements were made with a Perkin-Elmer MPF-44 spectrofluorometer equipped with a thermostated cell housing as described previously (Marsh & Lowey, 1980).

In addition, the tryptophan-fluorescence enhancement of IAF-labeled subfragment 1 in the presence of MgATP was found to be in the range 13–31% (excitation wavelength 300 nm, emission wavelength 340 nm; 10 mM Tris and 1.8 mM $MgCl_2$, pH 8.0, or 10 mM imidazole and 1.8 mM $MgCl_2$, pH 7.0).

Pre-Steady-State Kinetic Measurements. Measurements of the rate of the tryptophan-fluorescence enhancement were carried out in a stopped-flow apparatus as previously described (Chock & Eisenberg, 1979). For the IAF-fluorescence studies, the excitation wavelength used was 490 nm, and the emitted fluorescence was measured by using an Ealing 26-4333 cutoff filter which has 1.4% transmission at 490 nm and 52% transmission at 510 nm, where the emission intensity of the IAF-labeled S-1 is near its maximum value (Marsh & Lowey, 1980).

Results

Before investigating the nature of the kinetic step which causes the decrease in IAF fluorescence, we determined whether IAF-labeled S-1 shows the same ATP-induced change in tryptophan fluorescence as is observed with unlabeled S-1. Figure 1A shows the increase in tryptophan fluorescence of unlabeled S-1 when it is mixed with 200 μM ATP at pH 7.8, 15 °C. Figure 1B shows an identical experiment using S-1 labeled with IAF on the alkali light chain. In both of these experiments, the excitation wavelength was 300 nm and the

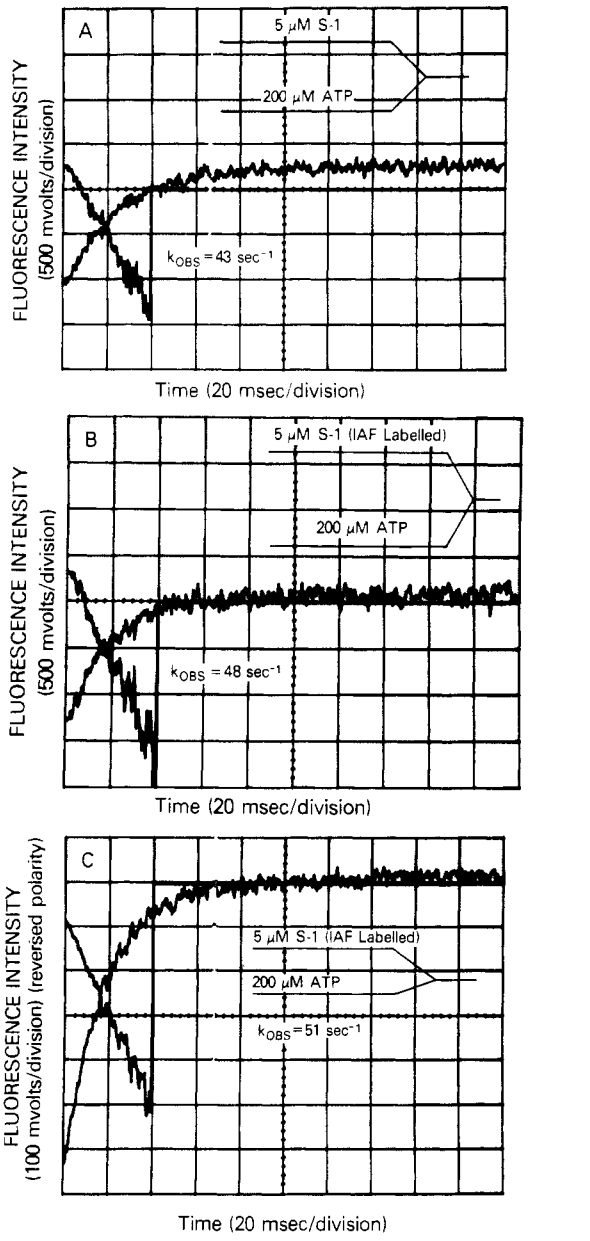

FIGURE 1: Time course of the fluorescence intensity change for S-1 immediately after the addition of ATP. In (A) unmodified S-1 is used while in (B) and (C), IAF-labeled S-1 is used. The fluorescence intensity measured in (A) and (B) is the tryptophan-fluorescence enhancement. The fluorescence intensity decrease measured in (C) (the trace here is inverted to facilitate calculation of a first-order rate constant) is the IAF-fluorescence intensity. See Materials and Methods for details. The superimposed linear plot in each portion of the figure is a computer semilog plot whose slope gives the first-order rate constant.

emission wavelength was 340 nm. With unlabeld S-1, the fluorescence trace yields a linear first-order plot with a rate constant of 43 s^{-1}, a value consistent with earlier studies on unlabeled S-1 (Chock et al., 1979). With IAF-labeled S-1, a nearly identical fluorescence trace is obtained with a rate constant of 48 s^{-1}. This suggests that, as was found for the steady-state ATPase activity, the initial P_i burst is unaffected by the fluorescence label on the alkali 1 light chain.

We next determined which step in the ATPase cycle is associated with the 6–10% IAF-fluorescence decrease observed when ATP is mixed with IAF-labeled S-1 (Marsh & Lowey, 1980). The two most likely possibilities are the ATP-binding step and the ATP-hydrolysis step. Our approach was to compare the kinetic properties of the fluorescence decrease

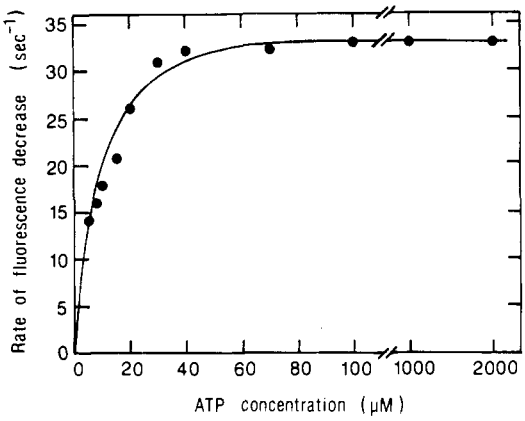

FIGURE 2: Rate constant of the IAF-fluorescence decrease of IAF-labeled S-1 as a function of ATP concentration. Conditions: 10 mM imidazole, pH 7.0, 15 °C, 1.8 mM $MgCl_2$, and 2.5 μM IAF-labeled S-1. The smooth curve is simply drawn by eye and does not represent a detailed fit of the data. The data, when plotted as a double reciprocal, do not give a linear plot and have a form similar to that found by Chock et al. (1979).

with the properties of the tryptophan-fluorescence increase which is known to be mainly caused by the ATP-hydrolysis step (Johnson & Taylor, 1978; Chock et al., 1979). We first studied the time course of the IAF-fluorescence decrease which was measured by using an excitation wavelength of 490 nm and an emission wavelength of 510 nm. The results of such an experiment, using the same preparation of IAF-labeled subfragment 1 that gave the data in Figure 1B, are shown in Figure 1C. Note that the fluorescence trace was inverted to facilitate calculation of the first-order rate constant; in reality, the fluorescence intensity decreased. This trace yields a linear first-order plot with a rate constant of 51 s^{-1}, showing that the decrease in IAF fluorescence (Figure 1C) follows the same time course as the increase in tryptophan fluorescence (Figure 1B). This suggests that the decrease in IAF fluorescence may be associated with the same kinetic step as the increase in tryptophan fluorescence.

Further evidence that this is the case comes from studies comparing the ATP dependence of the rates of the IAF-fluorescence decrease and the tryptophan-fluorescence increase. It has previously been shown that, as the ATP concentration is increased, the rate constant for the fluorescence increase levels off at a relatively low value, while the rate constant for irreversible ATP binding increases linearly, approaching very high values at millimolar concentrations of ATP (Chock et al., 1979). These data demonstrated that most of the tryptophan-fluorescence increase was associated with the ATP-hydrolysis step, rather than the irreversible ATP binding.

To determine if the decrease in IAF fluorescence is also due to the ATP-hydrolysis step, we studied the ATP dependence of the rate constant for this fluorescence decrease. This experiment was performed under conditions (pH 7, very low ionic strength) where the rate constant for irreversible ATP binding becomes greater than 100 s^{-1} at high ATP concentration. As shown in Figure 2, the results for IAF fluorescence are very similar to the data obtained for tryptophan fluorescence under similar conditions (Chock et al., 1979). At 40 μM ATP, the rate constant for the fluorescence decrease levels off at a value of about 30 s^{-1}, and it remains constant at this value up to 2 mM ATP. Since under similar conditions the rate of irreversible ATP binding does not level off, it appears likely that the decrease in IAF fluorescence is caused by the ATP-hydrolysis step, rather than by irreversible binding of ATP.

As another test of this conclusion, we compared the increase in tryptophan fluorescence with the decrease in IAF fluorescence as a function of actin. Stein et al. (1981) showed that, as the actin concentration is increased, there is an increase in the rate constant for the tryptophan-fluorescence change; furthermore, they showed that within the dead time of their apparatus the complex MT ⇌ AMT comes into rapid equilibrium. Hence, the rate of the fluorescence enhancement measured in the presence of actin is the rate of the ATP-hydrolysis step. On this basis, they concluded that the rate constant for the ATP-hydrolysis step is probably even faster when subfragment 1 is bound to actin than when it is dissociated from actin. Figure 3 shows the decrease in IAF fluorescence which occurs with the same preparation of IAF-labeled subfragment 1 in the presence and absence of 25 μM actin. The rate constant appears to be significantly faster in the presence of actin (43 s^{-1}) than in the absence of actin (18 s^{-1}). Figure 4 shows a plot of the rate constant for the decrease in IAF fluorescence as a function of actin concentration with a different preparation of IAF-labeled subfragment 1. With this particular preparation, the rate constant of the fluorescence decrease in the absence of actin was somewhat higher (28 s^{-1}) than that with the previous preparation (18 s^{-1}). (A similar difference was observed in the rate constants of the tryptophan-fluorescence increase.) Yet, here too, a significant increase in the rate constant occurred as the actin concentration was increased, reaching a value of 44 s^{-1} at 35 μM actin. These data strongly support our conclusion that the increase in tryptophan fluorescence and the decrease in IAF fluorescence are caused by the same kinetic step in the ATPase cycle.

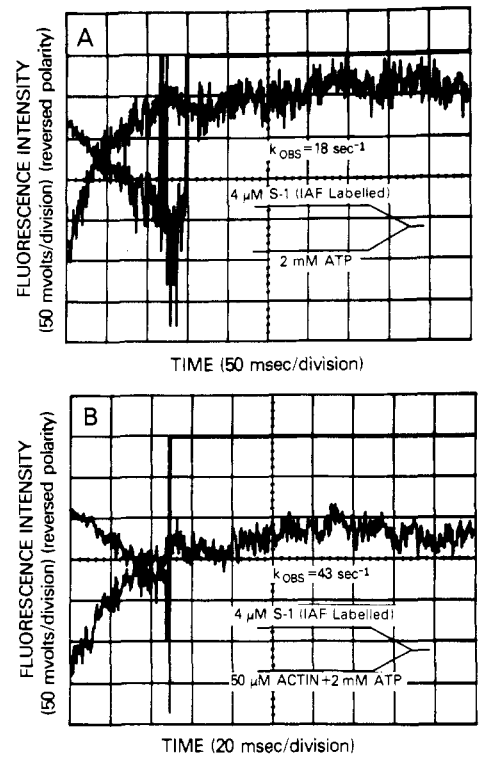

FIGURE 3: Effect of actin on the rate of the IAF-fluorescence decrease of IAF-labeled S-1 immediately after addition of ATP. (A) shows the time course of the fluorescence intensity change for IAF-labeled S-1 in the absence of actin. (B) shows a similar time course in the presence of 25 μM actin. The superimposed linear plot in each portion of the figure is a computer semilog plot whose slope gives the first-order rate constant. Conditions: same as in Figure 2, except ATP = 1 mM and IAF-labeled S-1 = 2 μM.

Discussion

Rate constants for the increase in tryptophan fluorescence of unmodified subfragment 1 and the decrease in extrinsic

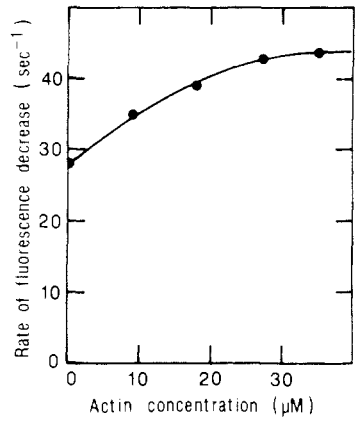

FIGURE 4: Rate constant of the IAF-fluorescence decrease of IAF-labeled S-1 as a function of actin concentration. A single preparation of IAF-labeled S-1 was used for this plot. Conditions: same as in Figure 3.

Table I: Comparison of the Rate of the Fluorescence Change for IAF-Labeled S-1 and Unmodified S-1

conditions	fluorescence rate (s^{-1}) unmodified[a] S-1 (λ_{ex} 300 nm, λ_{em} 340 nm)	IAF-labeled[b] S-1 (λ_{ex} 490 nm, λ_{em} 510 nm)
pH 7.0; 1 mM ATP[c]	23	18
pH 7.8; 0.1 mM ATP[d]	43	51
pH 7.8; 1 mM ATP[d]	51	58
pH 7.0; 1 mM ATP, 25 μM actin[c]	44	43

[a] The fluorescence change measured here is the tryptophan fluorescence. See Materials and Methods for details. [b] The fluorescence change measured here is the IAF-fluorescence decrease. See Materials and Methods for details. [c] Conditions: 10 mM imidazole, 1.8 mM $MgCl_2$, 15 °C. [d] Conditions: 10 mM Tris, 1.8 mM $MgCl_2$, 15 °C.

fluorescence of IAF-labeled subfragment 1 are summarized in Table I. The two sets of rate constants appear to change in parallel. In both cases, addition of F-actin, or a rise in pH from 7 to 7.8, increases the rate constant. Furthermore, both rate constants level off at a relatively low value as the ATP concentration is increased. Therefore, it appears that the ATP-hydrolysis step causes a conformational change which, in turn, induces both an increase in tryptophan fluorescence and a decrease in IAF fluorescence.

Flexibility of the myosin head or S-1 has long been inferred from electron microscopy (Slayter & Lowey, 1967; Elliott & Offer, 1978) and from the susceptibility of the head–rod junction to proteolysis (Lowey et al., 1969). Recent evidence from NMR (Highsmith et al., 1979) and thiol cross-linking studies (Wells & Yount, 1979; Wells et al., 1980) has supported this view of S-1 as a highly flexible, mobile molecule with at least the potential for large-scale structural changes.

Our finding that a fluorescent probe attached to a single cysteine residue near the C terminus of the alkali 1 light chain is able to sense the cleavage of ATP is consistent with such a dynamic structure. The fact that the rate constant for the burst is the same whether it is measured from the change in fluorescence emitted by the extrinsic chromophore on the light chain or from the intrinsic tryptophan on the heavy chain is all the more remarkable considering that a distance of more than 50 Å has been shown to exist between the fluorophore and the nucleotide-binding site by energy transfer (Moss & Trentham, 1979). We do not know the location of the tryptophan(s) on the heavy chain, but it has generally been assumed to be near the active site. The possibility of transmitting information over such large distances emphasizes, once again, the unusually flexible nature of the myosin head. This property may be related to the ability of ATP to greatly reduce the binding of actin to S-1, even though the actin and nucleotide-binding sites may be widely separated on the myosin head. It could also allow mechanical information to be directly transmitted to the ATP-active site, thus allowing feedback between force generation and ATPase activity in vivo.

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References

Bagshaw, C. R., & Trentham, D. R. (1974) *Biochem. J. 141*, 331–349.

Bagshaw, C. R., Eccleston, J. F., Eckstein, F., Goody, R. S., Gutfreund, H., & Trentham, D. R. (1974) *Biochem. J. 141*, 351–364.

Chock, S. P., & Eisenberg, E. (1979) *J. Biol. Chem. 254*, 3229–3235.

Chock, S. P., Chock, P. B., & Eisenberg, E. (1979) *J. Biol. Chem. 254*, 3236–3243.

Elliott, A., & Offer, G. (1978) *J. Mol. Biol. 123*, 505–519.

Fraser, A. B., Eisenberg, E., Kielley, W. W., & Carlson, F. C. (1975) *Biochemistry 14*, 2207–2214.

Highsmith, S., Akasaka, K., Konrad, M., Goody, R., Holmes, K., Wade-Jardetsky, N., & Jardetsky, O. (1979) *Biochemistry 18*, 4238.

Johnson, K. A., & Taylor, E. W. (1978) *Biochemistry 17*, 3432–3442.

Lowey, S., Slayter, H. S., Weeds, A. G., & Baker, H. (1969) *J. Mol. Biol. 42*, 1–29.

Marsh, D. J., & Lowey, S. (1980) *Biochemistry 19*, 774–784.

Moss, D. J., & Trentham, D. R. (1979) *Proc. Int. Congr. Biochem., 11th*, 203.

Slayter, H. S., & Lowey, S. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 1611–1615.

Stein, L. A., Chock, P. B., & Eisenberg, E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1346–1350.

Wells, J. S., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.

Wells, J. A., Knoeber, C., Sheldon, M. C., Werber, M. M., & Yount, R. G. (1980) *J. Biol. Chem. 255*, 11135–11140.

Werber, M. M., Szent-Gyorgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2883.