A Reaction Involving Protein Sulfhydryl Groups, a Bound Spin-Label, and $K_3Fe(CN)_6$ as a Probe of Sulfhydryl Proximity in Myosin†

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ABSTRACT: The EPR spectrum of myosin labeled with an iodoacetamide spin-label shows components corresponding to weakly and to strongly immobilized labels. Upon treatment of this myosin wiith $K_3Fe(CN)_3$, a chemical reaction involving the nitroxide group of the spin-label, $Fe(CN)_6^{3-}$, and a sulfhydryl group of the protein results in the selective destruction of the weakly immobilized spectral component without loss of ATPase activity. The involvement of a sulfhydryl group in this reaction is indicated by the fact that $K_3Fe(CN)_6$ destroys the electron paramagnetic resonance (EPR) signal of a spin-label only in the presence of a thiol-containing compound and that the signal is not lost if the sulfhydryl group is blocked. In the presence of MgADP, both the strongly immobilized component, attributable to labels bound to the SH-1 thiols of myosin, and the weakly immobilized component are lost on adding $K_3Fe(CN)_6$. The loss of the strongly immobilized component can be prevented by blocking the SH-2 thiols, a result that is consistent with the view [Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. F. (1974) *Biochemistry 13*, 3837–3840] that SH-1 is near SH-2 in the presence of MgADP. Coincident with the loss of the strongly immobilized component is the loss of the Ca^{2+}-activated ATPase activity, indicating that the SH-2 thiol is chemically modified and therefore participates in the reaction. These results suggest the potential use of $K_3Fe(CN)_6$ as a reagent for selective destruction of specific components of the EPR spectrum of a nitroxide-labeled protein or membrane, as a probe for the proximity of a thiol group and a nitroxide label, and, together with a nitroxide, as a reagent for chemical modification of protein sulfhydryl groups.

The EPR[1] spectrum of myosin labeled with the spin-label *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide shows components corresponding to weakly and strongly immobilized labels (Seidel et al., 1970). During the course of our studies on spin-labeled myosin, we observed the selective destruction of the weakly immobilized spectral component on incubation with $K_3Fe(CN)_6$. This phenomenon could not be explained by previous studies which have shown that $K_3Fe(CN)_6$ acts as an oxidizing agent, preventing or reversing the loss of signal brought about by chemical reduction of the spin-label (Kaplan et al., 1973; Ross & McConnell, 1975; Quintanilha & Packer, 1977), nor could it be accounted for in terms of paramagnetic broadening by $K_3Fe(CN)_6$, which results in the loss of spectral amplitude (Likhtenshtein et al., 1970; Grebenshchikov et al., 1971; Reichman et al., 1972; Likhtenshtein, 1976; Keith et al., 1977; Morse, 1977; Berg et al., 1979).

We decided to study this $K_3Fe(CN)_6$-induced loss of the EPR signal of spin-label for several reasons: first of all, to gather new information on the chemistry of nitroxide spin-labels, secondly, to gain further insight into the substructure of the myosin molecule, and, finally, because of the potential usefulness of the selective removal of certain components of the spectra of spin-labeled proteins. The selective removal of weakly immobilized spectral components, as illustrated here in the case of myosin, may be particularly useful in studying the rotational motion of proteins by saturation transfer EPR, when the spin-label should be rigidly bound so that it accurately reflects the motion of the protein (Thomas, 1978).

Methods and Materials

SH-Modified Myosin. Procedures described previously were used in the preparation of myosin from rabbit muscle (Nauss et al., 1969). Modification of SH-1 sulfhydryl groups of myosin was carried out with 4 mol of iodoacetamide per mol of myosin or 2 mol of the spin-label, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide, per mol of myosin, as described previously (Seidel et al., 1970). For the purpose of blocking the SH-2 groups, myosin spin-labeled at SH-1 sulfhydryls was reacted with 4 mol of *N*-ethylmaleimide per mol of myosin in the presence of MgADP (Yamaguchi & Sekine, 1966). Myosin spin-labeled at SH-1 sulfhydryls was also reacted with *N*-ethylmaleimide in the absence of MgADP, in which case SH-2 sulfhydryls are not modified (Yamaguchi & Sekine, 1966). All modifications were carried out at 0 °C in a solution containing 20–40 mg of myosin per mL, 0.5 M KCl, 40 mM Tris, pH 8.0, and 1 mM EDTA or 1 mM MgADP. After 30–45 min, the reaction mixture was exhaustively dialyzed vs. 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7.0, at 4 °C to remove unreacted label.

The modification of SH-1 and SH-2 groups of myosin was monitored by following the K^+- and Ca^{2+}-activated ATPase activities (Table I). Modification of SH-1 sulfhydryls results in the loss of the K^+-activated activity and a marked increase in the Ca^{2+}-activated activity in 0.5 M KCl with a smaller increase in 0.05 M KCl, while subsequent modification of SH-2 sulfhydryls results in the loss of the Ca^{2+}-activated

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[1] Abbreviations used: EPR, electron paramagnetic resonance; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; M-IAA, myosin modified at SH-1 thiols with iodoacetamide; M-SL, myosin modified at SH-1 thiols with *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide spin-label; M-SL-MalNEt(ADP), M-SL modified at SH-2 thiols with *N*-ethylmaleimide in the presence of MgADP; M-SL-MalNEt, M-SL reacted with *N*-ethylmaleimide in the absence of MgADP; ASL, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)-acetamide; S and W, strongly and weakly immobilized EPR spectral components of spin-labeled myosin, respectively.

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Table I: ATPase Activity of SH-Modified Myosin

myosin	ATPase act. (μmol of P_i per mg of myosin per min) EDTA, 0.5 M KCl	Ca^{2+}, 0.05 M KCl	Ca^{2+}, 0.5 M KCl	modification by SH-1	SH-2
native myosin	1.48	1.24	0.38		
M-IAA	0.52		2.12	iodoacetamide	
M-SL	0.30	1.70	2.34	spin-label	
M-SL-MalNEt	0.08	1.31		spin-label	
M-SL-MalNEt(ADP)	0.08	0.16	0.57	spin-label	*N*-ethylmaleimide

activity (Kielley & Bradley, 1956; Yamaguchi & Sekine, 1966).

For the purpose of blocking all reactive sulfhydryl groups, myosin was reacted with a 500-fold molar excess of *N*-ethylmaleimide or a 5000-fold molar excess of iodoacetamide for 24 h. The reaction was carried out at 0 °C in a solution containing 1–10 mg of myosin per mL, 0.5 M KCl, 1 mM EDTA, and 2 mM Mops, pH 7 (for *N*-ethylmaleimide), or 0.1 M Tris, pH 8 (for iodoacetamide), and 6 M guanidine hydrochloride (for iodoacetamide).

Nitroxide Spin-Labels. The iodoacetamide spin-label, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)iodoacetamide, and the acetamide spin-label, *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)acetamide (ASL), were synthesized by standard methods (McConnell & Hamilton, 1968).

The fluorescent spin-label dansyl nitroxide, 1-(dimethylamino)naphthalene-5-[*N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)sulfonamide], was prepared by reported methods (Stryer & Griffith, 1965). All experiments with dansyl nitroxide were conducted in the dark since, in aqueous solution, light induced the loss of fluorescence and the EPR signal. A minimum concentration of 2% acetone was necessary to keep dansyl nitroxide in solution.

EPR Measurements. EPR spectra were recorded at room temperature with a Varian E-109E or V-4502 X-band spectrometer operating at 100-kHz field modulation. Samples were stored at 0 °C between measurements.

Fluorescence Measurements. Fluorescence measurements were made by employing a Perkin-Elmer MPF-4a spectrometer operating in the ratio mode and thermostated at 25 °C. Fluorescence of dansyl nitroxide was measured at 500 nm, the wavelength of maximal emission, while exciting at 320 nm, the wavelength of maximal excitation. The wavelengths of maximal emission and excitation did not change under the conditions used in these experiments. The fluorescence was corrected for absorbance of the exciting light by multiplying the fluorescence by the factor $10^{A/2}$, where A is the absorbance at the exciting wavelength.

ATPase Assay. ATPase activities were measured in a solution containing 0.05 M Tris (pH 7.5), 5 mM ATP, and 0.1–0.2 mg of myosin per mL. For K^+-ATPase the assay system also contained 0.5 M KCl and 5 mM EDTA and for Ca^{2+}-ATPase, 10 mM $CaCl_2$ and 0.5 or 0.05 M KCl. After incubation at 25 °C for 5 min, the reaction was stopped with an equal volume of 10% trichloroacetic acid and P_i determined by the method of Fiske & Subbarow (1925).

Results

The low-field region of the EPR spectrum of myosin labeled with iodoacetamide spin-label shows components corresponding to weakly (W) and strongly (S) immobilized labels (Seidel et al., 1970; Figure 1A). When this spin-labeled myosin is treated with dithiothreitol, which, like other thiols, is known to reduce nitroxides to diamagnetic hydroxylamines (Morrisett & Drott, 1969; McConnell & McFarland, 1970; Buckman, 1970; Giotta & Wang, 1972; Seidel, 1973; Baldassare et al., 1974), there is a partial loss of S and almost complete loss of W (Figure 1B). In an attempt to regenerate the initial spectrum, the dithiothreitol was removed by dialysis and $K_3Fe(CN)_6$, a mild oxidizing agent, was added. Within 3 min S returned to its initial amplitude (Figure 1C) which did not change on standing overnight. W returned to ~75% of its initial amplitude in 3 min (Figure 1C) and then decreased to almost zero on standing overnight (Figure 1D) and was not restored on removal of $K_3Fe(CN)_6$ by dialysis. When $K_3Fe(CN)_6$ was added to spin-labeled myosin without pretreatment with dithiothreitol, essentially the same results were obtained; the amplitude of S was not affected, but that of W

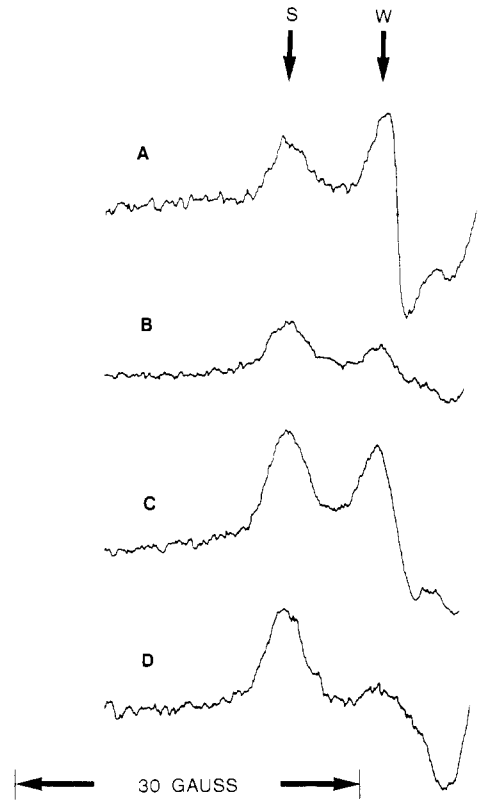

FIGURE 1: Low-field portion of the EPR spectrum of spin-labeled myosin (M-SL), 10 mg/mL, in a solution containing 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7. S and W indicate the spectral components corresponding to the strongly and weakly immobilized spin-labels, respectively. (A) Untreated; (B) treated overnight with 10 mM dithiothreitol and dialyzed exhaustively against 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7; (C) sample B followed by a 3-min incubation with 10 mM $K_3Fe(CN)_6$; (D) sample C followed by an additional 16-h incubation with 10 mM $K_3Fe(CN)_6$. The spectrum of (D) was not changed after removal of $K_3Fe(CN)_6$ by exhaustive dialysis against 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7. For (A) a relative gain setting of 1.0 and a relative gain response time of 0.3 were used. For (B)–(D) a relative gain setting of 1.6 and a relative gain response time of 1.0 were used.

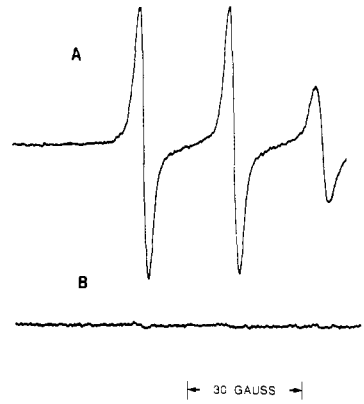

FIGURE 2: EPR spectrum of spin-labeled myosin (M-SL), 10 mg/mL, in a solution containing 4 M guanidine hydrochloride, 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7, (A) without $K_3Fe(CN)_6$ and (B) 2 min after adding 1 mM $K_3Fe(CN)_6$.

Table II: Effects of $K_3Fe(CN)_6$ or $NaIO_4$ on the EPR Signal of ASL in the Presence of Proteins, Amino Acids, or Thiols[a]

reagent	oxidant	% of signal amplitude remaining after addn of oxidant: after 2 min	after 1 h
myosin	$K_3Fe(CN)_6$ or $NaIO_4$	2	
bovine serum albumin	$K_3Fe(CN)_6$	30	
bovine serum albumin + 4 M guanidine hydrochloride	$K_3Fe(CN)_6$	0	
gelatin	$K_3Fe(CN)_6$	93	93
gelatin + 4 M guanidine hydrochloride	$K_3Fe(CN)_6$	93	93
tryptophan	$K_3Fe(CN)_6$	93	93
cysteine	$K_3Fe(CN)_6$ or $NaIO_4$	2	
dithiothreitol	$K_3Fe(CN)_6$	5	
β-mercaptoethanol	$NaIO_4$	0	

[a] The reaction medium contained 2×10^{-6} M ASL, 0.5 M KCl, 0.2 M Mops, and 1 mM EDTA, pH 7.0. Proteins were present at 10 mg/mL, tryptophan, dithiothreitol, and β-mercaptoethanol were present at a concentration of 1 mM, and cysteine was present at a concentration of 0.1 mM. ASL was added just before initiating the reaction by addition of the oxidant to a final concentration of 1 mM.

decreased almost to zero and was not restored on removal of $K_3Fe(CN)_6$. If spin-labeled myosin is denatured with guanidine hydrochloride, all labels become weakly immobilized, as indicated by a simple three-line spectrum, and $K_3Fe(CN)_6$ produces essentially complete loss of the spectrum within 2 min (Figure 2).

For determination of whether covalent binding of the spin-label to the protein was necessary for the loss of signal, $K_3Fe(CN)_6$ was added to an unbound label, ASL. The EPR spectrum of ASL consists of three lines of roughly equal amplitude, characteristic of a freely rotating spin-label. In the presence of myosin the EPR spectrum of ASL was lost almost completely within 2 min after adding $K_3Fe(CN)_6$ (Table II), while in the absence of myosin there was an immediate reduction of signal amplitude of only 7%, apparently due to paramagnetic broadening, with no further loss on standing overnight, showing that the label need not be bound to the protein but that the protein is essential for loss of signal. Bovine serum albumin could replace myosin since $K_3Fe(CN)_6$ induced a rapid loss of signal in the presence of guanidine

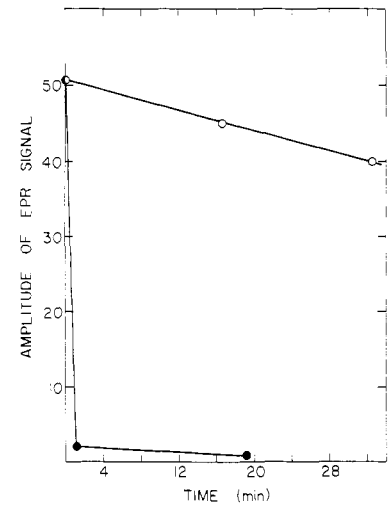

FIGURE 3: Destruction of EPR signal of ASL by cysteine or cysteine plus $K_3Fe(CN)_6$. The solution contained 2.5×10^{-6} M *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)acetamide in 0.5 M KCl, 0.2 M Mops, and 1 mM EDTA, pH 7. EPR signal amplitude is plotted as a function of time after adding (○) 1 mM cysteine or (●) 1 mM cysteine plus 1 mM $K_3Fe(CN)_6$.

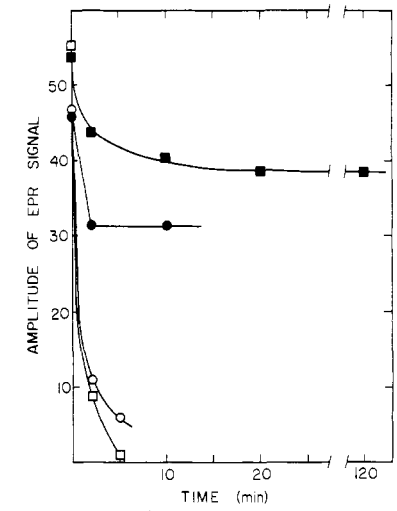

FIGURE 4: Effect of blocking myosin sulfhydryl groups with a large excess of sulfhydryl reagent on the destruction of the EPR signal of ASL by $K_3Fe(CN)_6$. The amplitude of the EPR signal was measured as a function of time after adding $K_3Fe(CN)_6$ to a solution of *N*-(2,2,6,6-tetramethyl-4-piperidinyl-1-oxy)acetamide (ASL) in 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7, in the presence of the following: (○) myosin, 10 mg/mL; (□) myosin, 1 mg/mL, and 4 M guanidine hydrochloride; (●) *N*-ethylmaleimide-blocked myosin, 10 mg/mL; (■) iodoacetamide-blocked myosin, 1 mg/mL, and 4 M guanidine hydrochloride. In the presence of guanidine hydrochloride the solutions contained 2.5×10^{-6} M ASL and 1 mM $K_3Fe(CN)_6$ was added, and in the absence of guanidine hydrochloride the solutions contained 2.5×10^{-5} M ASL and 10 mM $K_3Fe(CN)_6$.

hydrochloride and a slower loss in the absence of guanidine hydrochloride; gelatin, however, which lacks the amino acids tryptophan and cysteine, produced no loss of signal (Table II). Tryptophan could not replace myosin or bovine serum albumin, producing no loss of the signal on addition of $K_3Fe(CN)_6$, but there was an immediate loss of signal in the presence of cysteine, or other thiols, and $K_3Fe(CN)_6$ (Table II). When cysteine is reacted with *N*-ethylmaleimide, $K_3Fe(CN)_6$ no longer induces loss of the ASL signal (data not shown). This effect of cysteine is not due to direct reduction of the nitroxide by the thiol group because cysteine alone produces a much slower loss of signal (Figure 3). To verify the involvement of cysteine residues of the protein in the loss of signal, we investigated the effect of blocking the sulfhydryl groups of

Table III: Effect of $K_3Fe(CN)_6$ on the Strongly (S) and Weakly (W) Immobilized EPR Spectral Components of Spin-Labeled Myosin in the Presence of Cysteine[a]

time after adding cysteine ± $K_3Fe(CN)_6$	amplitude of EPR spectral components on adding: cysteine S	cysteine W	cysteine + $K_3Fe(CN)_6$ S	cysteine + $K_3Fe(CN)_6$ W
0	4.0	6.0	4.0	6.0
5 min			2.7	1.0
25 min			2.1	0.7
3.5 h			1.7	0.2
26 h	4.0	2.7	0.0	0.0

[a] 1 mM cysteine was added to a solution containing 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7, and 10 mg of spin-labeled myosin (M-SL) per mL. To half of this solution was added $K_3Fe(CN)_6$ to a final concentration of 10 mM, and the EPR spectra of each half were recorded at the times indicated.

Table IV: Effect of $K_3Fe(CN)_6$ on the Strongly Immobilized Spectral Component (S) of Spin-Labeled Myosin in the Presence or Absence of ADP[a]

myosin	amplitude of S remaining 24 h after adding $K_3Fe(CN)_6$ (%): −ADP	+ADP
M-SL	100	34
M-SL-MalNEt(ADP)	100	86
M-SL-MalNEt	100	20

[a] 10 mM $K_3Fe(CN)_6$ was added to spin-labeled myosin (30 mg/mL) in a solution containing 0.5 M KCl, 40 mM Mops, and 10 mM $MgCl_2$, pH 7.0, in the presence or absence of 10 mM ADP. $K_3Fe(CN)_6$ results in the loss of the weakly immobilized component for all samples.

myosin. In the presence of myosin that had been reacted with a large excess of *N*-ethylmaleimide or iodoacetamide, the rate of $K_3Fe(CN)_6$-induced loss of signal of ASL is greatly reduced (Figure 4). All of these results strongly indicate that the $K_3Fe(CN)_6$-induced loss of the nitroxide EPR signal in the presence of protein involves a cysteine residue.

$K_3Fe(CN)_6$ should also destroy the strongly immobilized signal of spin-labeled myosin in the presence of added cysteine, provided the strongly immobilized labels are accessible to the solvent, which has been shown by previous studies (Seidel, 1973; Onishi & Morales, 1976). On addition of $K_3Fe(CN)_6$ to spin-labeled myosin in the presence of cysteine, both spectral components are lost within 26 h (Table III). Cysteine alone has no effect on the strongly immobilized component under conditions used in this experiment, though there is a slow loss in the weakly immobilized component. Thus, the spin-label need not be weakly immobilized or freely mobile for $K_3Fe(CN)_6$ to induce loss of signal. However, the loss of signal depends primarily on the proximity of the spin-label to a sulfhydryl group. In view of these results, our initial observation of the $K_3Fe(CN)_6$-induced loss of the weakly immobilized spectral component of spin-labeled myosin indicates that a cysteine residue is near the weakly immobilized label.

Reisler et al. (1974) have shown that the SH-1 and SH-2 thiols of myosin can be cross-linked in the presence of MgADP, suggesting that they are close or become close on adding MgADP. We reasoned that, if so, then for spin-labeled myosin the strongly immobilized component of the EPR spectrum arising from labels bound to SH-1 groups (Seidel et al., 1970) might be destroyed by $K_3Fe(CN)_6$ in the presence of MgADP, which is known to make SH-2 groups reactive (Yamaguchi & Sekine, 1966). Treatment of spin-labeled myosin with $K_3Fe(CN)_6$ does destroy most of the strongly immobilized component of the EPR spectrum in the presence of MgADP but not in its absence (Table IV). MgADP produces no loss of EPR signal in the absence of $K_3Fe(CN)_6$. Evidence for the participation of SH-2 groups in the loss of the strongly immobilized component is obtained from experiments in which SH-2 groups of spin-labeled myosin are blocked with *N*-ethylmaleimide in the presence of MgADP. In this case very little loss of the strongly immobilized component occurs in the presence of $K_3Fe(CN)_6$ and MgADP (Table IV), even though this doubly labeled myosin still binds MgADP, as indicated by a slight shift in the position of the strongly immobilized component (Seidel et al., 1970; Seidel, 1972). On the other hand, SH-2 groups are not blocked when the reaction with *N*-ethylmaleimide is carried out in the absence of ADP as indicated by the persistence of the Ca^{2+}-activated ATPase activity (Table I). In this case, the results are the same as when there has been no treatment with *N*-ethylmaleimide; in the presence of MgADP, $K_3Fe(CN)_6$ destroys most of the strongly immobilized component (Table IV). These results indicate the specific involvement of SH-2 thiols in the $K_3Fe(CN)_6$-induced loss of EPR signal of spin-labels attached to SH-1 thiols and are consistent with the view that SH-1 thiols are close to SH-2 thiols in the presence of MgADP (Reisler et al., 1974).

Changes in enzymatic activity also accompany the loss of EPR signal of spin-labeled myosin in the presence of $K_3Fe(CN)_6$ and MgADP. The Ca^{2+}-activated ATPase is decreased under these conditions (Table V), a change characteristic of the modification of SH-2 groups (Yamaguchi & Sekine, 1966). In the absence of ADP, neither activity (Table V) nor the strongly immobilized component (Table IV) was lost. The essential role of the nitroxide in the modification of SH-2 groups is illustrated by the fact that $K_3Fe(CN)_6$ has no effect on the ATPase activities of native or SH-1 iodoacetamide-modified myosin, in the presence or absence of MgADP (Table V). Thus, the SH-2 groups appear to be chemically modified in a reaction with the nitroxide and $K_3Fe(CN)_6$.

The possibility that the nitroxide and the thiol group become covalently linked in this reaction was tested by using an excess of myosin as a source of thiol groups and a fluorescent nitr-

Table V: Effect of $K_3Fe(CN)_6$ on the ATPase Activity of Spin-Labeled Myosin in the Presence or Absence of ADP[a]

myosin	ATPase act. (μmol of P_i per mg of myosin per min): no addition, EDTA, 0.5 M KCl	no addition, Ca^{2+}, 0.05 M KCl	no addition, Ca^{2+}, 0.5 M KCl	$K_3Fe(CN)_6$ treatment, EDTA, 0.5 M KCl	$K_3Fe(CN)_6$ treatment, Ca^{2+}, 0.05 M KCl	$K_3Fe(CN)_6$ treatment, Ca^{2+}, 0.5 M KCl	$K_3Fe(CN)_6$ + ADP treatment, EDTA, 0.5 M KCl	$K_3Fe(CN)_6$ + ADP treatment, Ca^{2+}, 0.05 M KCl	$K_3Fe(CN)_6$ + ADP treatment, Ca^{2+}, 0.5 M KCl
native	1.48	1.24	0.38	1.36	1.15		1.34	1.18	
M-SL	0.30	1.70	2.34	0.26		2.25	0.15	0.39	0.64
M-IAA	0.52		2.12	0.51		1.95	0.44		2.05

[a] $K_3Fe(CN)_6$ was added to a final concentration of 10 mM to a solution containing 0.5 M KCl, 40 mM Mops, and 10 mM $MgCl_2$, pH 7.0, and myosin at 30 mg/mL, in the presence or absence of 10 mM ADP. After 24 h at 0 °C, $K_3Fe(CN)_6$ was removed by exhaustive dialysis against a solution containing 0.5 M KCl, 2 mM Mops, and 1 mM EDTA, pH 7.0, before the ATPase assay was performed.

Table VI: Dansyl Nitroxide Fluorescence of the Supernatant of a Reaction Mixture of Dansyl Nitroxide, Insoluble Myosin, and $K_3Fe(CN)_6$[a]

sample	EPR signal remaining after 24 h (%)	fluorescence of supernatant remaining after 24 h (%)
dansyl nitroxide	100	100
dansyl nitroxide + $K_3Fe(CN)_6$	94	80
dansyl nitroxide + myosin	97	103
dansyl nitroxide + myosin + $K_3Fe(CN)_6$	2	72

[a] Dansyl nitroxide at 3×10^{-5} M was in a solution containing 0.2 M KCl, 20 mM Mops, and 1 mM EDTA, pH 6.8. When myosin was present, its concentration was 10 mg/mL. $K_3Fe(CN)_6$ was added to a final concentration of 1 mM. After sample incubation in ice for 24 h, the EPR spectrum, which consists of three lines of roughly equal amplitude, was recorded, the myosin, if present, was then removed by centrifugation at 40000 rpm for 1 h, and the fluorescence (λ_{ex} 320 nm and λ_{em} 500 nm) of the samples was measured. After the EPR spectrum was recorded and before fluorescence was measured, 1.2 mM sodium ascorbate was added to the samples containing $K_3Fe(CN)_6$ in order to reduce $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$, which has a lower extinction coefficient at the exciting wavelength and results in a smaller absorbance correction to the fluorescence. Sodium ascorbate has no effect on the fluorescence of dansyl nitroxide alone. All incubations and measurements were carried out in the dark.

oxide, dansyl nitroxide, to determine whether the loss of the EPR signal was accompanied by binding of the fluorescent dye to myosin. This experiment was carried out at a low ionic strength where myosin forms aggregates and can be readily removed by sedimentation. If a complex were formed between myosin and the fluorescent nitroxide, the fluorescence of the supernatant would decrease in proportion to the loss of the EPR signal. After addition of $K_3Fe(CN)_6$ the reaction was allowed to go to completion and when only 2% of the EPR signal remained, the supernatant still contained 72% of the initial fluorescence (Table VI). This decrease in fluorescence was also observed when myosin was omitted from the reaction mixture (Table VI). Thus, the experiment shows that no significant complex formation between nitroxide and thiol moieties accompanies the reaction with $K_3Fe(CN)_6$.

Since $K_3Fe(CN)_6$ is an oxidizing agent, other oxidizing agents were tested to see whether this property is responsible for the loss of EPR signal. Br_2 (10 mM), when added to a solution containing ASL (2.5×10^{-5} M), destroys the EPR signal immediately whether or not a thiol-containing compound is present (data not shown). This is consistent with a report that Br_2 oxidizes nitroxides, in CCl_4, to the diamagnetic oxammonium ion which undergoes decomposition in H_2O to unidentified products (Golubev et al., 1965). $NaIO_4$ (10 mM) alone, on the other hand, has no effect on the EPR signal of ASL, but it destroys the entire signal, almost immediately, when cysteine or other thiols are present (Table II). Thus, another oxidant, IO_4^-, can replace $Fe(CN)_6^{3-}$ in the reaction leading to the loss of the EPR signal.

Since $K_3Fe(CN)_6$ oxidizes cysteine and other thiols to disulfides (Bohning & Weiss, 1960; Kolthoff et al., 1962; Little & O'Brien, 1967; Bridgart et al., 1973), it was desirable to know what role, if any, thiol oxidation plays in the loss of the EPR signal. The influence of the rate of oxidation was investigated by changing pH since the rate increases with increasing pH (Kolthoff et al., 1962; Meehan et al., 1962; Bridgart et al., 1973; Bridgart & Wilson, 1973) and by including EDTA, which inhibits the oxidation (Gorin & Godwin, 1966; Bridgart & Wilson, 1973). The disappearance of the yellow color accompanying the reduction of $Fe(CN)_6^{3-}$ to $Fe(CN)_6^{4-}$ was used as a semiquantitative indicator of the oxidation rate. $K_3Fe(CN)_6$ was added to a solution containing ASL and a slight excess of cysteine; after 2 min the EPR signal was recorded and it was noted whether or not the yellow $K_3Fe(CN)_6$ color had faded (Table VII). In 2 min cysteine alone had negligible effects on the EPR signal (not shown). When the pH is decreased or EDTA is added, the oxidation rate decreases, as evidenced by the persistence of the yellow color, while, in general, the rate of disappearance of the EPR signal increases. As pH is decreased in the presence of EDTA, the rate of disappearance of the EPR signal first increases down to pH 5 and then begins to decrease on further reduction of the pH. The rate of oxidation of cysteine by $K_3Fe(CN)_6$ in the presence of EDTA also decreases at pH values below 5 (Bridgart & Wilson, 1973). Thus, the rapid loss of the EPR spectrum only occurred under conditions where the yellow color of $Fe(CN)_6^{3-}$ persisted, that is, where the oxidation rate is relatively slow; however, if the oxidation rate is decreased further then the rate of signal loss also decreases.

Table VII: Effect of $K_3Fe(CN)_6$ on the EPR Signal of ASL in the Presence of Cysteine as a Function of pH in the Absence and Presence of EDTA[a]

	−EDTA		+EDTA	
pH	signal amplitude after 2 min (%)	persistence of color	signal amplitude after 2 min (%)	persistence of color
3.5	0	+	28	+
5	64	±	0	+
6	100	−	6	+
7	100	−	4	+
8	100	−	100	−

[a] $K_3Fe(CN)_6$ was added to a final concentration of 0.9 mM to a solution containing 2.5×10^{-5} M ASL, 1 mM cysteine, 0.2 M buffer, and, when added, 1 mM EDTA. Cysteine was added just before $K_3Fe(CN)_6$ to minimize the reduction of ASL by cysteine. The buffers used were Tris at pH 8.0, Mops at pH 8.0, Mops at pH 7.0, 2-(*N*-morpholino)ethanesulfonic acid at pH 6.0, citric acid at pH 5.0, and formic acid at pH 3.5. The persistence of the yellow color of $Fe(CN)_6^{3-}$ after 2 min is indicated by +, complete loss of the yellow color is indicated by −, and a partial fading is indicated by ±. The color was estimated and the EPR spectrum recorded 2 min after addition of $K_3Fe(CN)_6$.

Discussion

The selective destruction by $K_3Fe(CN)_6$ of the weakly immobilized spectral component of spin-labeled myosin cannot be explained by the mechanism of paramagnetic broadening by $K_3Fe(CN)_6$ (Likhtenshtein, 1976; Keith et al., 1977), because the removal of $K_3Fe(CN)_6$ does not result in the reappearance of the lost signal. Therefore, the loss of signal must be attributed to a chemical reaction. Because $K_3Fe(CN)_6$ destroys the EPR signal of a nitroxide only in the presence of a thiol-containing compound and blocking of the thiol group prevents the loss of signal, this reaction must involve a thiol group. In the case of spin-labeled myosin, this thiol group is on a cysteine residue of the protein. The observation that guanidine hydrochloride accelerates the loss of the EPR signal in the presence of protein (Figure 2 and Table II) can be explained by the exposure in the denatured protein of cysteine residues, which are relatively inaccessible to the spin-label and to $K_3Fe(CN)_6$ in the native state. In view of the present results, in studies using $K_3Fe(CN)_6$ as a reagent to broaden nitroxide EPR signals by means of magnetic interactions in

order to assess the accessibility of spin-labels to $Fe(CN)_6^{3-}$ (Lepock et al., 1975; Robey et al., 1979), one must be sure that the loss of signal amplitude is not due to a chemical reaction involving a nearby SH group. This can be accomplished most simply by determining whether removal of $K_3Fe(CN)_6$ leads to a restoration of the signal.

Although experiments described here do not substantiate any one mechanism for the reaction involving nitroxide, thiol, and $K_3Fe(CN)_6$, certain possibilities are suggested and others are ruled out. The possibility of a covalent addition compound between the nitroxide and thiol is ruled out by the fact that the fluorescent spin-label, dansyl nitroxide, does not become bound to myosin when the EPR signal is lost. Nitroxides can be reduced to the diamagnetic hydroxylamines (Gaffney, 1976), but it seems reasonable to discount the reduction of the nitroxide as a possible mechanistic route for several reasons. Simple reduction of the nitroxide by the thiol (Morrisett & Drott, 1969; McConnell & McFarland, 1970; Buckman, 1970; Giotta & Wang, 1972; Seidel, 1973; Baldassare et al., 1974) cannot account for the loss of EPR signal since the signal of ASL is lost much more slowly in the presence of a thiol alone than when $K_3Fe(CN)_6$ is also present. In addition, the concentrations of oxidants, $K_3Fe(CN)_6$ or $NaIO_4$, used in these experiments were in substantial excess of the thiol and the products of reduction of nitroxides are readily reoxidized by $K_3Fe(CN)_6$ to the paramagnetic nitroxides (Kaplan et al., 1973; Ross & McConnell, 1975; Quintanilha & Packer, 1977; Figure 1).

Since another oxidizing agent, $NaIO_4$, can replace $K_3Fe(CN)_6$ in the loss of nitroxide EPR signal, oxidation appears to be involved in some way. Oxidation of nitroxide radicals has been achieved by both chemical (Golubev et al., 1965) and electrochemical (Serve, 1975; Sümmermann & Deffner, 1975) means. Br_2 or Cl_2 in CCl_4 oxidizes nitroxides to the diamagnetic oxammonium ion which undergoes decomposition in H_2O to unidentified products (Golubev et al., 1965). Br_2 also appears to oxidize the unbound spin-label, ASL, in H_2O, since the EPR signal is lost immediately on adding Br_2. However, neither $K_3Fe(CN)_6$ nor $NaIO_4$ oxidizes nitroxides directly. The inability of $NaIO_4$ to oxidize nitroxides appears to rest on kinetic rather than thermodynamic grounds, since $NaIO_4$ [redox potential = 1.4 V (Sober, 1968)] is a stronger oxidizing agent than Br_2 [redox potential = 1.1 V (Sober, 1968)]. The inability of $NaIO_4$ or $K_3Fe(CN)_6$ to oxidize nitroxides may be related to the anionic nature of the oxidizing species.

Other results suggest that the oxidation of the thiol by $K_3Fe(CN)_6$ plays a major role in the loss of the nitroxide EPR signal. In experiments designed to investigate the relationship between the loss of the EPR signal of ASL and the oxidation of cysteine by $K_3Fe(CN)_6$, it was found that, in general, as the oxidation rate decreased, the rate of the disappearance of the EPR signal increased. Furthermore, if the oxidation rate is decreased sufficiently then the rate of signal loss also decreases, suggesting the possibility that an intermediate in the $K_3Fe(CN)_6$ oxidation of cysteine reacts with the spin-label, resulting in the loss of the EPR signal.

Although the mechanism of the reaction involving nitroxide, cysteine, and $K_3Fe(CN)_6$ is not fully understood, the resulting loss of EPR signal can be very useful in selectively removing specific components of the EPR spectrum of nitroxide-labeled proteins. This depends, of course, on the favorable location of a cysteine residue with respect to the label whose signal is to be destroyed; however, when such a condition does not exist, then the addition of cysteine or other thiols and $K_3Fe(CN)_6$ provides a possible alternative method of removing the unwanted signal. The selective removal of the signal from weakly immobilized labels is of particular importance in studying the rotational motion of proteins by saturation transfer EPR, where the spin-label must be rigidly bound if it is to accurately reflect the motion of the protein (Thomas, 1978). $K_3Fe(CN)_6$ is a relatively mild reagent, and except for the loss of activity of spin-labeled myosin in the presence of MgADP it has little effect on the enzymatic activity of myosin.

The usefulness of $K_3Fe(CN)_6$ is also illustrated by the loss of the strongly immobilized spectral component of SH-1 spin-labeled myosin in the presence of MgADP and the concomitant modification of the SH-2 thiols reflected in the loss of Ca^{2+}-ATPase activity. These results indicate that the signal is lost through a reaction involving the spin-label, $K_3Fe(CN)_6$, and SH-2 thiols and are consistent with the view that the SH-1 thiols are close to the SH-2 thiols in the presence of MgADP (Reisler et al., 1974). Although the products of the reaction involving $K_3Fe(CN)_6$, nitroxide, and sulfhydryl groups have not been characterized, $K_3Fe(CN)_6$ can be used to chemically modify sulfhydryl groups in the presence of a nitroxide spin-label, as a reagent for selective destruction of specific EPR spectral components of a spin-labeled protein, and as a probe to detect cysteine residues close to a protein-bound spin-label.

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Carboxamidopeptidase: Purification and Characterization of a Neurohypophyseal Hormone Inactivating Peptidase from Toad Skin†

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ABSTRACT: Carboxamidopeptidase, an enzyme which inactivates neurohypophyseal hormones, has been purified 3800-fold in an overall yield of 22% from toad skin, a neurohypophyseal hormone target organ, by $(NH_4)_2SO_4$ fractionation, DEAE-Sephadex chromatography, and affinity chromatography on immobilized *p*-aminobenzamidine and concanavalin A-agarose. The purified enzyme is capable of inactivating both [8-arginine]vasopressin (AVP) and oxytocin by hydrolyzing the Arg^8–Gly^9–NH_2 and the Leu^8–Gly^9–NH_2 bonds, respectively, and can hydrolyze the ester substrates, benzoyl-L-arginine ethyl ester (BzArgOEt) and acetyl-L-tyrosine ethyl ester, suggesting that the enzyme has both trypsin-like and chymotrypsin-like activities. Carboxamidopeptidase is maximally active at pH 7.5-8.5 for AVP and BzArgOEt and pH 7.0 for oxytocin. Carboxamidopeptidase is inhibited by ovoinhibitor, ovomucoid, Trasylol, lima bean trypsin inhibitor, concanavalin A, antipain, leupeptin, chymostatin, elastatinal, *p*-nitrophenyl *p*-guanidinobenzoate, and 4-methylumbelliferyl *p*-guanidinobenzoate but not by soybean trypsin inhibitor, α_1-antitrypsin, hirudin, pepstatin, bestatin, phosphoramidon, or cysteine. The enzyme is also inhibited by the serine protease inhibitor, diisopropyl phosphofluoridate (*i*-Pr_2PF), and by the chloromethyl ketone derivatives of tosyllysine, tosylphenylalanine, and (benzyloxycarbonyl)phenylalanine, as well as by the sulfhydryl group reagent, *p*-(chloromercuri)benzoate (PCMB). Inhibition by PCMB is reversed by cysteine. The molecular weight determined by gel filtration in the presence of 1 M NaCl is approximately 100 000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicates that the enzyme is composed of two identical subunits of 48 000 daltons. Each subunit consists of a heavy chain (28 000 daltons) and a light chain (19 000 daltons) joined by a disulfide bond(s). Labeling experiments using [^{3}H]-*i*-Pr_2PF showed that the enzyme active site is located in the heavy chain.

A neurohypophyseal hormone inactivating peptidase, first detected (Campbell et al., 1965) in extracts of toad urinary bladder, a neurohypophyseal hormone target organ, was originally thought to be trypsin-like in its specificity since it hydrolyzed the Lys^8–Gly^9–NH_2 bond of [8-lysine]vasopressin (LVP)[1] with the release of free glycinamide. Subsequently, it was shown in this laboratory (Glass et al., 1969; Walter et al., 1972; Grzonka et al., 1974) that the toad bladder preparation could hydrolyze not only the Lys^8–Gly^9–NH_2 and Arg^8–Gly^9–NH_2 bonds of LVP and AVP, respectively, which are substrates for trypsin but also the Leu^8–Gly^9–NH_2 bond of oxytocin which is a substrate for chymotrypsin (Walter & Hoffman, 1974). Specificity studies using a variety of neurohypophyseal hormone analogues demonstrated that the en-

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[1] Abbreviations used: LVP, [8-lysine]vasopressin; AVP, [8-arginine]vasopressin; [^{14}C]AVP, [8-arginine,9-glycinamide-*1*-^{14}*C*]vasopressin; [^{14}C]oxytocin, [9-glycinamide-*1*-^{14}*C*]oxytocin; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester; CH-Sepharose 4B, Sepharose 4B coupled with 6-aminohexanoic acid; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; *i*-Pr_2PF, diisopropyl phosphofluoridate; [^{3}H]-*i*-Pr_2PF, [1,3-^{3}H]diisopropyl phosphofluoridate; Tos-Lys-CH_2Cl, tosyllysine chloromethyl ketone; Tos-Phe-CH_2Cl, tosylphenylalanine chloromethyl ketone; Z-Phe-CH_2Cl, (benzyloxycarbonyl)phenylalanine chloromethyl ketone; PCMB, *p*-(chloromercuri)benzoate; D-TrpOMe, D-tryptophan methyl ester; $NaDodSO_4$, sodium dodecyl sulfate; Me_2SO, dimethyl sulfoxide.

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