Sensitivity of Spin-Labeled Sarcoplasmic Reticulum to the Phosphorylation State of the Catalytic Site in Aqueous Media and in Dimethyl Sulfoxide†

Carol Coan

ABSTRACT: Under controlled conditions, the iodoacetamide spin-label is highly selective for the sarcoplasmic reticulum adenosinetriphosphatase (SR ATPase), labeling 7–8 nmol of reactive residues per mg of SR protein, or approximately two residues per active enzyme unit. The electron paramagnetic resonance (EPR) spectrum of the labeled enzyme exhibits a small but specific broadening on the binding of substrates or inorganic phosphate. Addition of Ca^{2+} greatly increases the substrate broadening but reverses the effect of P_i. Here we demonstrate that the Ca^{2+} effect on the enzyme–substrate spectrum is due to the division of the major spectral component into two components, each representing two distinct conformational environments which impart slightly different degrees of mobility to the spin-label. These components are not readily separated in the spectrum of the fully labeled enzyme, and stoichiometric labeling techniques are used to resolve the components and obtain splitting parameters. The two spectral components exhibit random redistribution on removal and reintroduction of substrate, indicating that they may represent two forms of a given site. E–P formation by P_i in the absence of Ca^{2+} does not produce two discernible components in the EPR spectrum, although a small broadening effect is apparent, and two components can be resolved in the reaction kinetics of *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide with SR. However, addition of dimethyl sulfoxide, which greatly facilitates phosphorylation of the enzyme, induces a two-component EPR spectrum. We conclude that a conformational change occurs in the enzyme specifically under conditions of high phosphate affinity which affects the motional parameters of 3–4 nmol of labeled residues per mg of SR, a number equal to the phosphorylation sites in our preparations. This affinity is induced by substrate and Ca^{2+} binding at activating sites in the normal mode of the enzymatic cycle, or by dimethyl sulfoxide in a general manner. In addition, we observe the conformational change with non-adenosine substrates in varying degrees, while 5′-adenylyl imidodiphosphate and adenosine 5′-(β,γ-methylenetriphosphate) are both fully effective. Since the latter analogues do not actively phosphorylate the enzyme, it is concluded that the conformational change is related to formation of a transition complex which is highly permissive of phosphoryl transfer. This complex is maximized for an adenosine moiety, while Ca^{2+} remains a stringent requirement.

The catalytic cycle of sarcoplasmic reticulum (SR)[1] ATPase includes a phosphorylated enzyme intermediate (Yamamoto & Tonomura, 1967; Makinose, 1969; Inesi et al., 1970) which is formed by incorporation of ATP-terminal phosphate onto an aspartyl residue of the enzyme protein (Bastide et al., 1973; Degani & Boyer, 1973). The cycle is then completed with hydrolytic cleavage and P_i release. In turn, the reverse direction of the cycle begins with P_i incorporation into the enzyme to form a phosphorylated intermediate (Masuda & de Meis, 1973) which, in appropriate experimental conditions, reacts with ADP to yield ATP (Hasselbach, 1978; de Meis & Vianna, 1979).

A most important difference in the requirement for enzyme phosphorylation in the forward or reverse direction of the cycle is that Ca^{2+} must be present for utilization of ATP, while it must be removed for the enzyme to become reactive to P_i (Masuda & de Meis, 1973; Chaloub et al., 1979). Since Ca^{2+} binds cooperatively to the enzyme (McIntosh & Berman, 1978; Inesi et al., 1980a; Verjovski-Almeida, 1981) independent of the substrate, it is likely that enzyme reactivity is subjected to allosteric regulation. In this connection, it was previously reported (Coan & Inesi, 1977) that the major component of the EPR spectrum of spin-labeled ATPase undergoes reversible changes upon addition of nucleotides or orthophosphate. On further addition of Ca^{2+}, the nucleotide effect on the spectrum is greatly increased while the orthophosphate effect is completely removed (Coan & Inesi, 1977).

In line with spectral observations, recently completed kinetic analysis of the spin-label reactivity with SR demonstrated two populations of reactive residues when substrate and Ca^{2+} were both bound to activating sites on the enzyme while one uniform population was observed under all other conditions (Coan & Keating, 1982). This suggested that the significant broadening effect of Ca^{2+} on the EPR spectrum may be due to the appearance of a second component representing the residues of increased reactivity. However, the reversibility of two components, following quenching of the reaction and removal of substrates, made isolation of a given component by standard procedures very difficult. Here we have developed stoichiometric labeling techniques so that each component may be observed directly as it labels in the sample cell. Characterization of the spectral components follows.

Further kinetic experiments now show that the addition of orthophosphate in the absence of Ca^{2+} introduces two populations of reactive residues, although two conformational environments are not distinct in the EPR spectrum in aqueous media. However, additions of Me_2SO, which greatly favor phosphorylation of the enzyme (de Meis et al., 1980), produce a two-component EPR spectrum. Taken together, the kinetic and spectral data demonstrate the sensitivity of the spin-labeled

† From the Department of Physiology and Biophysics, University of the Pacific Dental School, San Francisco, California 94115. *Received April 29, 1983.* Supported by National Institutes of Health Grant HL27867-01.

[1] Abbreviations: SR, sarcoplasmic reticulum; ATPase, adenosinetriphosphatase; Me_2SO, dimethyl sulfoxide; Mops, 4-morpholinepropanesulfonate; ISL, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide; AMP-P(NH)P, 5′-adenylyl imidodiphosphate; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); AcP, acetyl phosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate; ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); EPR, electron paramagnetic resonance; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-5826$01.50/0

SR spectrum to specific conformational states associated with the presence of a phosphate moiety at the active site of the enzyme.

Experimental Procedures

SR vesicles were prepared from the white skeletal muscle of rabbit hind legs by using methods previously described (Elter & Inesi, 1972). Vesicles were stored in a buffered sucrose medium (30% sucrose–10 mM Mops, pH 6.8) at 4 °C and were used within 3–4 days of preparation. *N*-(1-Oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide (ISL) was obtained from Syva. Spin-labeled protein was obtained by incubating 5 mg/mL SR with 0.4 mM ISL for 50 min at 25 °C in a medium containing 20 mM Mops–80 mM KCl, pH 6.8. The reaction was then quenched by addition of a large excess of cold medium and immediate centrifugation (42000*g* for 90 min). Excess ISL was removed by two repeated centrifugations, and the ISL-SR pellet was resuspended to give a final protein concentration of approximately 35 mg/mL. Aliquots of substrate, solvent, and cations were added immediately before each experiment as required. SR protein concentrations were determined by the techniques of Lowry et al. (1951) with bovine serum albumin as a standard.

EPR spectra were obtained with a JEOL-ME IX electron spin resonance spectrometer equipped with a variable temperature accessory. Protein samples were scanned over a 100-G range at 6-mW microwave power by using a modulation width of 1.60 G, a response time of 1 s, and a scan rate of 100 G/10 min. When comparative spectra were measured, aliquots of the same SR suspension were used, the spectrometer was adjusted with the first aliquot in place, and no further adjustments were made between samples. The same sample cell was used and was carefully repositioned between each measurement. Using these techniques, we could reproduce a given protein spectrum within a 3% variation. Viscous solvents such as Me_2SO and glycerol were added slowly under constant stirring on a vortex mixer immediately before use. In highly broadened spectra, where high-field maxima and minima are less distinct, given spectra were reproduced at greatly increased amplitudes, by using a modulation width of 2.0 G, a response time of 10 s, and a scan rate of 100 G/25 min. In addition, selected spectra were measured over a 200-G field range to sharpen the spectral features. Reproducible splitting parameters were obtained by these techniques.

The method of following the reaction kinetics of ISL and SR can be found in detail in Coan & Keating (1982). The rate equation is given by $\ln([SH]/[ISL]) = kt([SH]^0 - [ISL]^0) + \ln([SH]^0/[ISL]^0)$. The concentration of ISL was assumed to be directly proportional to the line height of the free label in solution. This in turn was monitored as the reaction progressed in the sample cell. The line height corresponding to the initial concentration of ISL, $[ISL]^0$, was determined from a standard sample identical in all respects with the reaction mixture, except that SR which had previously been fully reacted with nonlabeled iodoacetamide was used. At each given time, the concentration of SH residues reactive to ISL is given by $[SH] = [SH]^0 - ([ISL]^0 - [ISL])$, where $[SH]^0$ is determined from the end point of the reaction. When two classes of residues were present, the slope and intercept were determined for the slowest class, giving respectively the apparent rate constant and the number of residues in this class. The linear extrapolation of this slope was then subtracted from the experimental curve and the difference used to calculate a new kinetic plot for the remaining residues.

To start the labeling reaction, a volume of buffer containing ISL was added to an equal volume of SR, the final concentrations being 4×10^{-1} mM ISL, 5 mg/mL SR, 20 mM Mops, pH 6.8, and 80 mM KCl, at 25 °C. Cations and substrates were added as required to the initial SR suspension. The standard samples of ISL were prepared concurrently with each SR sample and measured in the sample cell with identical spectrometer settings; before each labeling experiment, several aliquots were then used to check signal reproducibility. All samples were scanned by using a modulation width of 1 G, a response time of 0.3 s, and a scan rate of 100 G/5 min. During the first part of each experiment, only the low-field lines were scanned so data points could be taken at 1-min intervals.

When stoichiometric labeling experiments were performed, small aliquots of ISL solutions were added directly to concentrated SR suspensions (60–80 mg of SR/mL). Due to the low concentration of label, a modulation width of 2 G was used in conjunction with a response time of 3 s and a sweep width of 100 G/10 min to monitor the labeled enzyme signal. Since slower reacting residues required extensive incubations for the labeling reaction to go to completion (4–5 h), the low-field component of the enzyme spectrum, which can be observed even in the presence of a large free label signal, was monitored over the duration of the incubation. This component did not change with time in the experiments given here, indicating that the enzyme conformation remained intact. Also, in slow labeling experiments, AMP-PCP was used. This analogue is considerably more resistant to hydrolysis by SR than AMP-P(NH)P and should exhibit little decomposition under the most stringent conditions used here (Taylor, 1981). This analogue also produces the same SR spectrum and kinetic partitioning effect as AMP-P(NH)P. Enzyme concentrations in different stoichiometric labeling solutions are not necessarily constant. Therefore, spectrometer amplitudes were adjusted to give signals of similar line height for purposes of comparison.

Protein spectra, obtained under differing conditions, are compared primarily on the basis of the hyperfine splitting parameter, $2T_{\parallel}$, taken as the separation of the outermost components in the spectrum. This parameter is related to the molecular rotation of the spin-label residues in the magnetic field, an increase in splitting being indicative of a lesser degree of molecular motion (McConnell & MacFarland, 1970). Although correlation times may be calculated from this parameter for highly ordered systems, these calculations cannot be applied to protein residues unless a specific axis of rotation can be defined (Stryer & Griffith, 1965; Grill et al., 1982). However, it can be said that the anisotropic mobility observed in the spectra here is in a range attributable to the segmental flexibility of labeled residues rather than rotation or translational movement of the enzyme as a whole (Thomas, 1978). Consequently, this parameter has served as a qualitative comparison of protein conformational change in many studies (Marsh, 1981; Berliner, 1976), and it may be assumed that, in the absence of other environmental factors, a significant change in $2T_{\parallel}$ is directly related to a change in the molecular environment around the labeled residues that either further restricts or increases the molecular motion of the spin-label in accordance with the direction of change in the parameter.

When changes in a given spin-labeled enzyme spectrum are monitored as a function of time or reagent concentration, two low-field lines in the spectrum, which are highly sensitive to the particular change being monitored, are chosen such that one increases in magnitude while the other decreases as the change progresses. The ratio of the two lines then gives a large numerical value which can be followed with accuracy in small increments. The direction of change of this ratio is not related

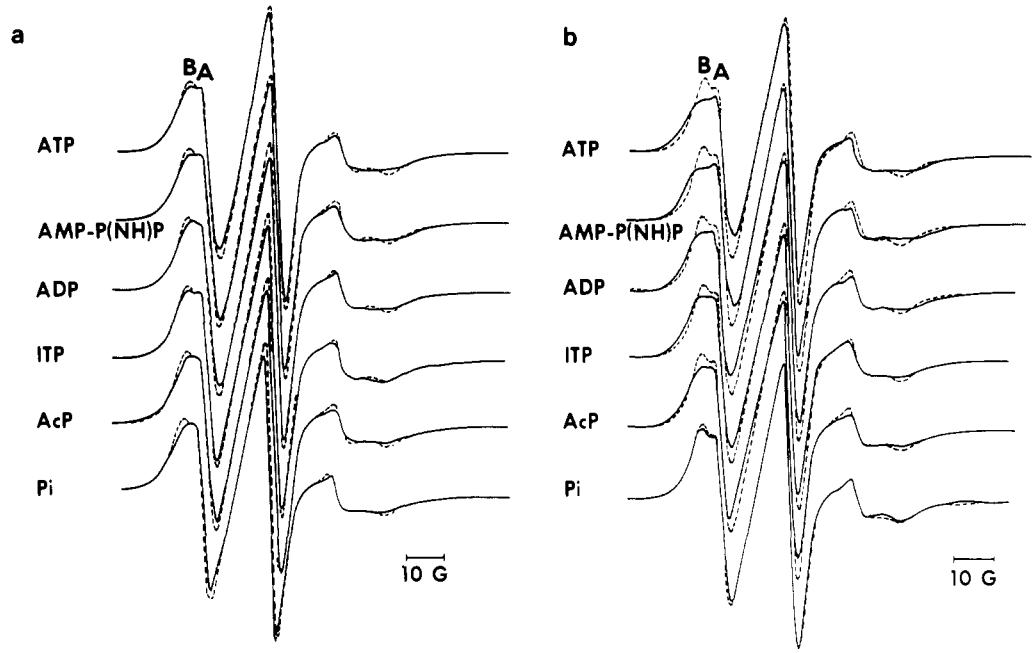


FIGURE 1: EPR spectra of ISL-SR in the absence (dashed lines) and in the presence (solid lines) of various substrates: (a) in the absence of Ca^{2+}; (b) with the addition of Ca^{2+}. The medium consisted of 20 mM Mops (pH 6.8), 80 mM KCl, 20 mM $MgCl_2$, 20 mM substrate, and 30–35 mg of SR protein/mL. EGTA (2 mM) was added to (a), and $CaCl_2$ (5 mM) was added to (b). The temperature was 25 °C. Samples were prepared from equal aliquots of a given SR solution. Line heights were reproducible to within 3%.

to changes in molecular parameters.

Results and Discussion

Spectral Effects of ATPase Substrates and Ca^{2+}. The spectra of ISL covalently bound to SR ATPase are complex and includes at least two components (Figure 1), as is often observed in covalently labeled proteins (Morrisett, 1976; Marsh, 1981). In early spin-label studies of SR (Landgraff & Inesi, 1969; Inesi & Landgraff, 1970), it was observed that small modifications of the spectra are produced by nucleotides in the absence of Ca^{2+}. Comparative spectra with various substrates of the SR ATPase are shown in Figure 1a, and it is evident that a slight effect is produced in all cases, including P_i. Since this effect is very small, it is difficult to relate it to parameters of enzyme activation. Much greater spectral effects are found when nucleotides are added in the presence of Ca^{2+} (Figure 1b), which is a required cation for enzyme activation. No significant effects are produced on ISL-SR spectra by Ca^{2+} in the absence of substrates under the conditions used here.[2]

Under controlled conditions, the iodoacetamide spin-label is highly selective, labeling 7–8 nmol of reactive residues per mg of SR protein or approximately two residues per active enzyme unit as defined by the number of moles of actively phosphorylated enzyme obtained at a given time (Coan & Keating, 1982). It has previously been demonstrated that the covalently bound labels reside on the ATPase protein (Champeil et al., 1978) and that the labeled enzyme retains activity at levels nearly identical with those of the nonlabeled enzyme. The small "mobile" component in the EPR spectrum (labeled A in the low-field lines in Figure 1), although discernible in every case, can be attributed to a small portion of the labeled sites. From spectral integrations of simulated two-component spectra, we estimate that this mobile component accounts for approximately 2% of the total signal intensity. This component is slow reacting and may be due to a different class of labeled residues, or to denaturation of a small amount of protein, but shows no relation to substrate or Ca^{2+} binding. A similar mobile component is present in the spectrum of maleimide-labeled SR (Thomas & Hidalgo, 1978) and has been attributed to a small number of reactive residues on proteins other than the ATPase (Hildalgo & Thomas, 1977). In any case, the major component in the iodoacetamide spectrum, labeled B in Figure 1, can be attributed to most of the 7–8 nmol of label. Additions of Ca^{2+} in the presence of nucleotide produce a broadening of this component accompanied by an apparent increase in the hyperfine splitting parameters. The Ca^{2+} concentration dependence of enzyme activation and spectral effects are identical (Coan & Inesi, 1977).

An important feature that we wish to point out here is that the spectral change is largest for ATP, AMP-P(NH)P, and ADP. Considering that ATP is a substrate used at very high rates, AMP-P(NH)P is an analogue used at extremely low rates (Taylor, 1981), and ADP is not utilized by the SR ATPase, it is apparent that the observed spectral effects are not dependent on substrate utilization but rather on the substrate presence at the catalytic site of the Ca^{2+}-activated enzyme. As AMP is not effective, we conclude that a maximum of two phosphate groups is also necessary, in addition to adenosine.

Smaller spectral effects are produced by inosine 5′-triphosphate (ITP) and acetyl phosphate which are also substrates for the SR ATPase, suggesting a relationship between the structure of the substrate and the magnitude of the spectral effect. No change is produced by P_i in the presence of Ca^{2+}.

We conclude that the occurrence of spectral changes in the Ca^{2+}-activated enzyme requires a polyphosphate substrate and is maximized by a favorable structure of the non-phosphate moiety of the substrate (e.g., adenosine).

Kinetic and Spectral Analysis of the Ca^{2+}-Induced Effect. It was recently shown that a Ca^{2+}-induced conformational change in the SR ATPase increases by 10-fold the reactivity

[2] At low temperatures, a clearly discernible change in the EPR spectrum with Ca^{2+} binding has been well characterized (Champeil et al., 1976). At 25 °C, this change is small and falls within our experimental reproducibility.

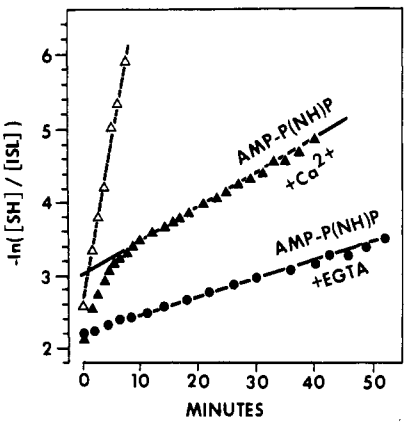


FIGURE 2: Second-order kinetic plot for the reaction of SR ATPase with ISL in the presence of AMP-P(NH)P with EGTA (●) or with $CaCl_2$ (▲); solid lines give the linear regression fit to the data between $t = 15$ and $t = 40$ min. For the AMP-P(NH)P data with $CaCl_2$, a kinetic plot of the faster reacting residues (△) has been obtained by graphical techniques given under Experimental Procedures. The medium was composed of 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM AMP-P(NH)P, 5 mM $MgCl_2$, 5 mM EGTA or $CaCl_2$, 0.4 mM ISL, and 5 mg/mL SR at 25 °C.

Table I: Number of Labeled Sulfhydryl Residues and Rate Constants for the Reaction of SR ATPase with ISL[a]

additions[b]	N_{fast}[c]	k_{fast}[d]	N_{slow}[c]	k_{slow}[d]	N_{total}[c]
AMP-P(NH)P·Mg + Ca	3.4	(50)	4.0	(7.5)	7.4
AMP-P(NH)P·Mg + EGTA	0.6		6.6	(8.8)	7.2
P_i·Mg + EGTA	3.3	(33)	3.7	(8.1)	7.0
Mg + EGTA	0.4		7.0	(5.6)	7.4
Me_2SO (40%) + P_i·Mg + EGTA	3.6	(53)	3.9	(2.9)	7.5
Me_2SO (40%) + Mg + EGTA	0.2		7.2	(2.5)	7.4

[a] The number of labeled residues (N) and rate constants (k) for each class were determined from the intercepts and slopes, respectively, of second-order kinetic plots as described under Experimental Procedures. At least five experiments were averaged for a given set of conditions. [b] Solutions contained the following: SR, 5.0 mg/mL; ISL, 0.4 mM; Mops, 20 mM (pH 6.8); KCl, 80 mM; $MgCl_2$, 5 mM (where applicable); P_i (with water), 20 mM; P_i (with Me_2SO), 5 mM; EGTA, 2 mM; $CaCl_2$, 5 mM; AMP-P(NH)P, 5 mM. [c] In nanomoles of ISL per milligram of SR; standard deviation ± 0.5. [d] In nanomoles of ISL per milligram of SR per minute × 10.

of half of the labeled sites with ISL, provided substrate is present (Coan & Keating, 1982). These are, of course, the conditions under which the major change is produced in the EPR spectrum. The kinetics are demonstrated in Figure 2 for AMP-P(NH)P and SR with and without additions of Ca^{2+}. The same effect is observed for AMP-PCP. Kinetic parameters are given in Table I, and it is apparent that 3–4 nmol of residues/mg of SR protein, a number equal to the phosphorylation sites in our preparations, is affected by the change, while another 3–4 nmol remains unaffected.

The fact that two populations of reactive residues are evident in the labeling kinetics has suggested that the broadened band in the EPR spectrum, induced by Ca^{2+}, may consist of two components with similar hyperfine splitting parameters which are difficult to discern in the spectrum of the fully labeled enzyme. However, isolation of the enzyme with one component uniquely labeled has proven difficult due to the requirement for prolonged substrate saturation through repeated washings.

We have avoided isolation difficulties by stoichiometrically labeling the enzyme directly in the EPR sample chamber. In this manner, a given spectral component may be observed

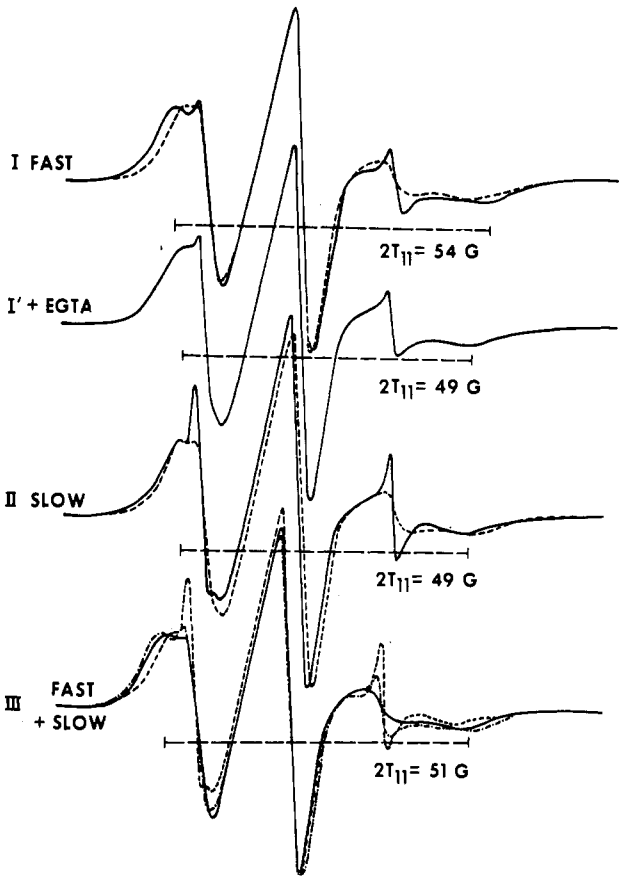


FIGURE 3: EPR spectra for SR ATPase stoichiometrically labeled with ISL in the presence of AMP-PCP·Mg and Ca^{2+}. (I) Fast labeling component obtained by addition of 2 nM ISL/mg of SR protein (—). The spectrum of AMP-PCP·SR without Ca^{2+} is given for comparison (---). (I′) Fast labeling component after addition of 10 mM EGTA. (II) Slower labeling component obtained by preincubation with 6 nM iodoacetamide; otherwise as in (I). (III) Fully labeled ISL-SR (—) is compared to fast (-·-·) and slow (---) labeling components. Splitting parameters, $2T_{\parallel}$, are given for solid-line spectra. For the spectra without Ca^{2+} (---) in (I) and (II), $2T_{\parallel}$ is 48.5 G. Spectra were adjusted to approximately equal line heights for comparative purposes. The medium was composed of 20 mM Mops (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 5 mM AMP-PCP, 5 mM $CaCl_2$, and 60 mg of SR protein/mL, 25 °C.

directly as the ISL reacts with the enzyme. Here, approximately 2 nmol of ISL/mg of SR is added to a high concentration of protein, and as the reaction progresses, the free ISL signal is fully reduced as the protein signal appears. The rate constants of the two label populations predict that 90% of the label will be directed to the faster reacting population at these concentrations. The resulting spectrum of 2 nmol of ISL/mg of SR (AMP-PCP and Ca^{2+} present) is given in Figure 3. It is clear from the figure that the more reactive residues produce a spectral component with increased hyperfine splitting ($2T_{\parallel}$ = 54 G vs. 48.5 G for the enzyme without Ca^{2+}), indicating that these residues are in a more constrained environment. A mobile component is always present in the spectrum of stoichiometric labeling reactions due to very small amounts of unreacted ISL, in addition to component A.

Most importantly, additions of EGTA directly to the sample cell on completion of the labeling reaction revert the EPR spectrum to a form similar in all respects to that observed without Ca^{2+}. This clearly demonstrates that the more reactive component is specifically sensitive to the effect Ca^{2+} induces in the spectrum.

On the other hand, when the enzyme is first incubated with 5–6 nmol of iodoacetamide (not spin-labeled) per mg of SR,

and then the ISL is added directly to the sample, the resulting spectrum primarily represents the slower reacting residues. As shown in Figure 3, II, the spectral properties of these residues are clearly distinct from those of the faster reacting residues and appear similar in terms of both splitting parameters and reaction rates (Table I) to the substrate–enzyme complex formed without Ca^{2+}. As would be predicted, additions of EGTA directly to the sample cell evoke little change in the spectrum.

It is apparent then that the broadened band of the Ca^{2+}·substrate·ATPase spectrum actually consists of two components, both representing fairly immobilized spin-labels, which are not distinctly separated in the fully labeled enzyme spectrum. A comparison of the fast and slow reacting components to the fully labeled enzyme is also given in Figure 3. These components are distinct from the small mobile component A, and the more constrained of the two is responsible for the Ca^{2+}-induced effect. Accordingly, it may be assumed that the Ca^{2+}-induced component represents a population of residues which are sensitive to a conformational change in the enzyme, which in turn makes these residues more reactive to ISL and more restricted in molecular motion when labeled. A second population of residues remains unaffected.

It is also interesting to compare the stoichiometry of the Ca^{2+}-induced effect to that of enzyme phosphorylation by substrate in the forward direction. The conditions used here in the spectral analysis are those which lead to maximal phosphorylation when ATP is used as a substrate, which, in fact, gives at most 4 nmol of E–P/mg of SR (Inesi et al., 1980a). This suggests that the 3–4-nmol fraction of responsive residues may be related to a unique conformation of the enzyme corresponding to proper utilization of the phosphorylating substrate for which Ca^{2+} binding is a required factor.

Effect of P_i on ISL Reaction Kinetics. While nucleotides are effective substrates which are utilized by the SR ATPase by transfer of their terminal phosphate onto an aspartyl residue (Bastide et al., 1973; Degani & Boyer, 1973) of the enzyme, P_i, which is the product of hydrolytic cleavage of the phosphorylated enzyme intermediate, can be utilized in a reverse direction of the enzyme cycle (de Meis & Vianna, 1979). Accordingly, additions of P_i in the absence of Ca^{2+} produce a stable form of the enzyme intermediate (Masuda & de Meis, 1973). Reactivity of the enzyme to P_i under these conditions is low, however.

Kinetic studies of the reactivity of ISL with the ATPase were performed in the presence of 20 mM orthophosphate (EGTA also present). A biphasic behavior identical with that shown in Figure 2 was observed. Kinetic parameters from second-order plots are given in Table I. Intercepts from the plot indicate that a 3–4-nmol fraction of the sites increases in reactivity while another 3–4 nmol remains unaffected, as in the case with AMP-P(NH)P and Ca^{2+}. However, the rate constant of the faster labeling residues, k_{fast}, appears to be significantly lower than k_{fast} observed in the presence of AMP-P(NH)P and Ca^{2+}.

On the other hand, when P_i·SR is stoichiometrically labeled with ISL, both the faster and slower reacting residues exhibit the same EPR spectrum. And, of course, the spectrum does not show the broadening effect associated with Ca^{2+} activation. Consequently, it appears that to a certain degree the reactivity of the residues is sensitive to the presence of phosphate at the active site, while the division of the spectrum into two distinct conformational components requires the presence of a substrate and Ca^{2+} at activating sites under the conditions used here.

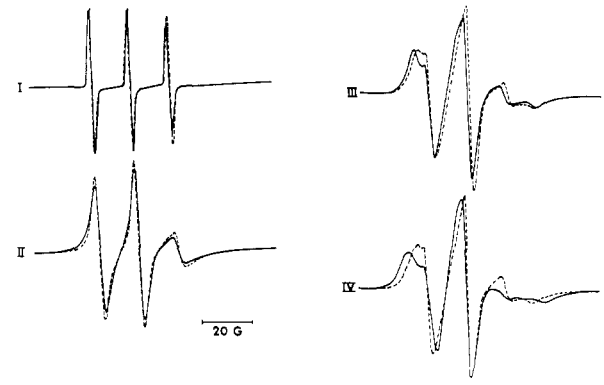


FIGURE 4: Effect of Me_2SO and glycerol on EPR spectra of the ISL spin-label. (I) Free ISL (2×10^{-4} M) in totally aqueous medium (---) and in the presence of 40% (v/v) Me_2SO (—); (II) ISL-SR (denatured) in the absence (---) and in the presence (—) of 40% (v/v) Me_2SO (ISL-SR was denatured by the addition of detergent, 4% SDS); (III) ISL-SR, as in (II) except that no denaturing agent was added, and the spin-labeled enzyme remained functionally competent; (IV) as in (III) except that 40% (v/v) glycerol was used in place of Me_2SO. The medium consisted of 20 mM Mops (pH 6.8), 80 mM KCl, and 32 mg/mL protein (when present). The temperature was 25 °C.

Spectral Effects of Me_2SO and P_i. Formation of phosphoenzyme by incorporation of P_i is greatly facilitated by the presence of 30–40% (v/v) Me_2SO. The solvent effects on the various aspects of enzyme activity have been well characterized (The & Hasselbach, 1977), and although enzyme turnover is inhibited at these solvent concentrations, maximal levels of phosphoenzyme are found. In fact, enzyme inhibition is thought to be due in part to prevention of phosphoenzyme hydrolysis (de Meis et al., 1980, 1982). Since in aqueous solutions P_i produces only minimal changes on the spectra of ISL-labeled SR, a series of experiments were performed in the presence of Me_2SO, in order to produce conditions favoring P_i utilization for enzyme phosphorylation.

However, solvents of varying polarity and viscosity may have effects on the spin parameters of a given EPR spectrum (Seelig, 1970; Jost et al., 1971; Jost & Griffith, 1978), and some degree of solvent characterization is initially required. It is shown in Figure 4 that relevant concentrations (40% v/v) of Me_2SO do not affect appreciably the spectra of free ISL in solution, or of ISL-SR when the protein structure is unfolded by the addition of the ionic detergent sodium dodecyl sulfate (SDS). The very small decrease in the hyperfine coupling constant in the isotropic free label spectrum ($\simeq$0.5 G) and the highly mobile denatured enzyme spectrum ($\simeq$1.0 G) is similar to that previously observed for solvent polarity effects on the nitroxide label in Me_2SO (Griffith et al., 1974). Similarly, the small spectral broadening of the denatured enzyme spectrum is in line with that attributed to viscosity effects when intermediate amounts of glycerol are added to highly mobile spectra (Deal et al., 1971).

On the other hand, a definite effect of Me_2SO is noted when this solvent is added to ISL-SR in the absence of denaturing agents. The effect consists of an enhancement in the hyperfine splitting parameters in the prominent spectral component, with an increase in the separation of the outer spectral lines from 47 to 51 G. An analogous effect is obtained with 40% glycerol. It is apparent then that these solvents produce a general restriction in molecular motion of the labeled residues when the enzyme is in its native conformation. Titrations indicate that enzyme inhibition (The & Hasselbach, 1977; Inesi et al., 1980b) occurs concomitantly with solvent perturbations to the spectrum, and these effects may well be due to changes in enzyme conformation around the labeled sites. Viscosity ef-

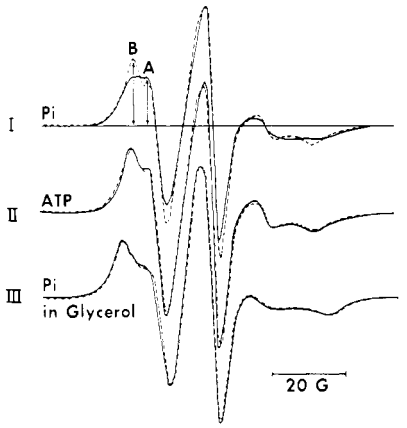


FIGURE 5: Effect of P_i in the absence of Ca^{2+}. (I) ISL-SR spectra in the absence (---) and in the presence (—) of 5 mM P_i and 5 mM $MgCl_2$. The medium consisted of 20 mM Mops, pH 6.8, 80 mM KCl, 2 mM EGTA, 35 mg of ISL-SR protein/mL, and 40% (v/v) Me_2SO. (II) As in (I) except that ATP (20 mM) was added in place of P_i. (III) As in (I) except that glycerol (40% v/v) was added in place of Me_2SO.

fects cannot be ruled out, however, since constrained anisotropic spectra may be more sensitive to changes in mobility than the highly mobile unfolded enzyme, or free label spectrum, as evidenced by the significant glycerol effect. Solvent polarity effects on anisotropic spectra are similar in direction and magnitude to those observed for the isotropic case (Seelig & Hasselbach, 1971) and therefore, would be expected to be very small. It is most likely that to some degree both viscosity and conformation are responsible for the solvent effect on the native SR.

Most importantly, however, the addition of P_i under conditions permitting phosphorylation of the enzyme (40% Me_2SO and Ca^{2+} concentration $<10^{-8}$ M) produces a large additional effect on the main component of the ISL-SR spectra (Figure 5). This effect consists of an apparent broadening of the spectral lines, accompanied by an increase in $2T_{\parallel}$ from 51 to 55 G. Little effect is produced by ATP under these conditions, or by P_i when glycerol is substituted for Me_2SO (Figure 5). These findings are consistent with the lack of ATP utilization in the absence of Ca^{2+} and with the fact that glycerol does not favor P_i utilization as Me_2SO does (de Meis et al., 1980; Watanabe et al., 1981). The unique dependence on conditions favoring phosphorylation suggests that the observed spectral broadening is also related to a specific enzyme conformation, as has been demonstrated for phosphorylating substrates in aqueous media.

Kinetic and Spectral Analysis of Phosphorylation in Me_2SO. The rate of reaction of ISL with SR ATPase in Me_2SO is similar to that found in aqueous media. Parameters obtained from second-order kinetic plots are given in Table I. Again, additions of P_i, in the presence of EGTA, increase the reactivity of a portion of the residues. The rate increase is approximately 20-fold, and intercepts from the kinetic plots indicate 3–4 nmol of sites/mg of SR becomes more reactive. On the other hand, when P_i is not present, uniform reactivity is observed. This is analogous to the effect of phosphorylation on the ISL reactivity in aqueous media. It should be noted, however, that k_{fast} is significantly higher in Me_2SO than in aqueous media with orthophosphate. This is in line with the increased reactivity of P_i with the SR in Me_2SO.

The unique broadening of the EPR spectrum by P_i also suggests that the two kinetic populations may introduce a second immobilized component into the spectrum in this solvent. Accordingly, the stoichiometric labeling experiments

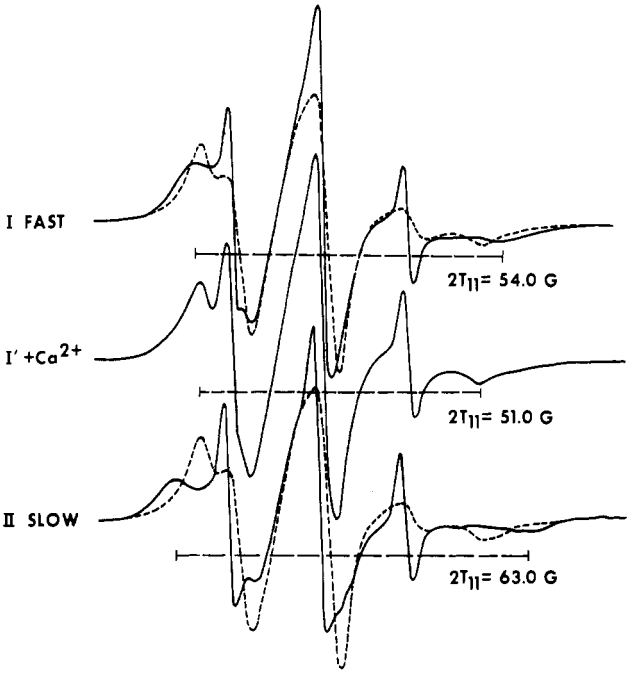


FIGURE 6: EPR spectra for SR-ATPase stoichiometrically labeled with ISL in Me_2SO. (I) Fast labeling component obtained by addition of 2 nM ISL/mg of SR protein to medium with P_i and EGTA. (I') Spectrum I after addition of 10 mM $CaCl_2$. (II) Slow labeling component obtained by preincubation with iodoacetamide. Dashed-line spectra in (I) and (II) compare ISL-SR without P_i. The medium was composed of 20 mM Mops (pH 6.8), 80 mM KCl, 40% (v/v) Me_2SO, 5 mM EGTA, 5 mM $MgCl_2$, and 5 mM P_i, 25 °C. Splitting parameters, $2T_{\parallel}$, are given for solid-line spectra. For the spectrum without P_i (---), $2T_{\parallel}$ is 51.0 G (not shown).

were repeated in Me_2SO with P_i and EGTA.

The spectrum obtained for the fast reacting component is given in Figure 6. Again, the faster reacting residues give a broadened spectrum. It is also known that additions of Ca^{2+} induce decay of the phosphoenzyme in Me_2SO as well as in aqueous media (de Meis et al., 1980). Accordingly, when Ca^{2+} is added to the stoichiometrically labeled enzyme, the EPR spectrum reverts to a form identical with that obtained without additions of P_i. Thus, it appears that the fast reacting component is related to conditions of phosphorylation.

On the other hand, when the enzyme is first labeled with plain iodoacetamide and then with ISL, the slow reacting component appears highly constrained. This is in contrast to that observed in aqueous media and may be due to aggregation following a long period of incubation. As a control, the spectrum was monitored as a function of time during the labeling process, and in selected experiments, a large excess of ISL was added to decrease the incubation time. In both cases, the low-field lines are separated from the large free ISL signal, and no apparent change was observed. However, a 1-h preincubation with iodoacetamide was still required, and some changes may occur during this time. This component showed no change on additions of Ca^{2+}.

It is apparent from the stoichiometric labeling experiments that a unique enzyme conformation is introduced by phosphorylation in Me_2SO. Again, the number of altered sites corresponds to the number of phosphorylation sites obtained. It is also apparent that in Me_2SO the presence of a nucleoside moiety is not required. This in turn points to the sensitivity of the long-observed spectral broadening effects to a specific conformational state associated with enzyme phosphorylation. This state appears to be obtained either through enzyme phosphorylation in the forward direction by substrate in

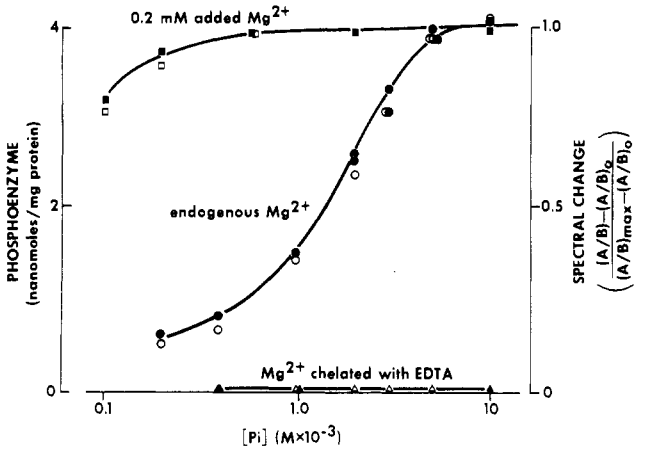


FIGURE 7: Parallel dependence of enzyme phosphorylation (○, □, △) and spectral changes (●, ■, ▲) on P_2 and Mg^{2+} concentrations. The reaction mixture consisted of 20 mM Mops (pH 7.0), 80 mM KCl, 40% (v/v) Me_2SO, and 1.0 mg of SR protein/mL for the phosphorylation reaction and of 34 mg of ISL-SR for EPR spectroscopy. The P_i concentration was as indicated. The reaction was allowed to proceed in the presence of endogenous Mg^{2+} (○, ●) or after addition of 0.2 mM $MgCl_2$ (□, ■) or after chelation of endogenous Mg^{2+} with 2 mM EDTA (△, ▲). The temperature was 37 °C. An empirical spectral parameter expressing the fractional change in the line height ratio A/B (as indicated in Figure 5) is used for a comparative evaluation of the spectral change. $(A/B)_0$ is the initial line height ratio and $(A/B)_{max}$ the final value.

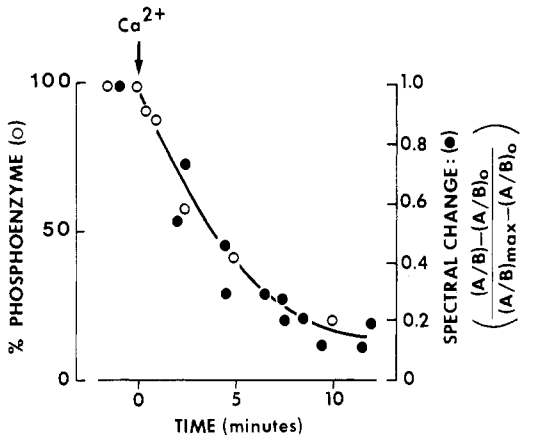


FIGURE 8: Ca^{2+}-induced phosphoenzyme decay and reversal of spectral changes. Phosphoenzyme was formed in reaction mixtures containing 30 mM Tris–maleate (pH 7.0), 10.0 mM $MgCl_2$, 2 mM EGTA, 2 mM P_i, and 10 mg of SR protein/mL for the phosphorylation experiments and containing 32 mg of ISL-SR/mL for the spectroscopic measurements. The temperature was 37 °C. Decay of the phosphoenzyme was initiated by the addition of 5 mM $CaCl_2$, and acid-quenched samples were collected at serial times for phosphoenzyme determinations. In parallel experiments, repeated EPR scans were obtained following the addition of Ca^{2+} over a limited portion of the spectrum including the downfield lines of the two spectral components. See the legend of Figure 11 for the definition of the empirical spectral parameter.

aqueous media or through phosphorylation by orthophosphate in the presence of Me_2SO.

A question then arises as to the lack of evidence of a conformational change in aqueous media on phosphorylation by P_i, particularly since two reactive populations are discernible in the labeling kinetics. It may be possible for solvent effects to enhance a small conformational change not directly discernible in the more mobile enzyme spectrum. In this light, the glycerol data have increased significance. This solvent is more viscous, and has a similar dielectric constant to Me_2SO, the solvent effects on the native enzyme spectrum being very similar. However, glycerol does not impart a high P_i sensitivity to the enzyme, and a large excess of P_i is required to maximize phosphorylation (de Meis et al., 1980). We find that P_i·Mg concentrations up to 50 mM do not show spectral evidence of a conformational change, although these concentrations should fully phosphorylate the enzyme in the low sensitivity state. It appears then that the change observed in the EPR spectrum is related to a conformational state associated with high phosphate sensitivity.

Phosphate Specificity. It should be pointed out that concentrated SR suspensions in Me_2SO, such as those used for EPR spectroscopy, contain sufficient endogenous P_i and Mg^{2+} to sustain phosphorylation when the reaction mixture is enriched with only one of the two. This is due to the effect of Me_2SO in rendering the SR ATPase very reactive to P_i (de Meis et al., 1980). And, in fact, a significant degree of spectral broadening can be seen when only $MgCl_2$ is added to the sample. That this can be attributed to the binding of endogenous phosphate is established in Figure 7.

When the reaction mixture is enriched with Mg^{2+}, full phosphorylation and spectral effects are observed if 0.1 mM P_i is present. This is approximately equal to the ATPase phosphorylation site concentration (25 mg of SR/mL) and is close to the levels of endogenous phosphate we found in our preparations at these concentrations. The unique specificity for P_i is then demonstrated by limiting the Mg^{2+} concentration. As shown in the figure, when only endogenous Mg^{2+} is present, 10 mM P_i must be added to fully phosphorylate the enzyme. The spectral broadening effect follows the phosphorylation curve directly. Finally, when the endogenous Mg^{2+} is chelated with EDTA, no effect is observed due to the stringent requirement of the phosphorylated enzyme for this cation.

As demonstrated in Figure 8, upon Ca^{2+} addition to a mixture maintaining an equilibrium level of phosphoenzyme, the phosphoenzyme decays to negligible levels. The reversal of the spectral broadening on Ca^{2+} addition to spin-labeled E–P is demonstrated in Figure 6. Figure 8 then demonstrates that both phosphoenzyme level and spectral change decay in parallel.

The two experiments shown in Figures 7 and 8 clearly establish that the spectral broadening observed in the EPR spectrum in Me_2SO is due specifically to enzyme phosphorylation rather than solvent effects or Mg^{2+} binding. This is particularly evident in the latter case, when P_i and Mg^{2+} are present in the medium, enzyme phosphorylation has been eliminated by Ca^{2+}, and the spectrum is virtually identical with that observed in Me_2SO without any cations, P_i, or substrates.

Reversibility of Components in the ISL-SR Spectrum. Observations of two constrained components in the ISL-ATPase spectrum when substrates and activators are present also introduce another interesting point. Kinetic studies of enzyme reactivity with ISL under these conditions have shown the two reactive populations of residues to exhibit properties of random redistribution on removal and reintroduction of substrate (Coan & Keating, 1982). This was observed when the enzyme reaction was quenched at given time intervals, washed, and reinitiated with fresh aliquots of AMP-P(NH)P and Ca^{2+}. Both fast and slow labeling populations reappeared in the kinetics, in equal proportion, even when all of the fast reacting residues should have been labeled initially, and only slow residues should have remained if the two populations were independent. Furthermore, the EPR spectrum of the labeled enzyme appeared to be identical irrespective of the time of incubation and gave what we see here to be the two-component spectrum. Both these experiments indicate that the enzyme reverts to an original "unbound" conformation on washing and then returns on rebinding of substrate and Ca^{2+} to a confor-

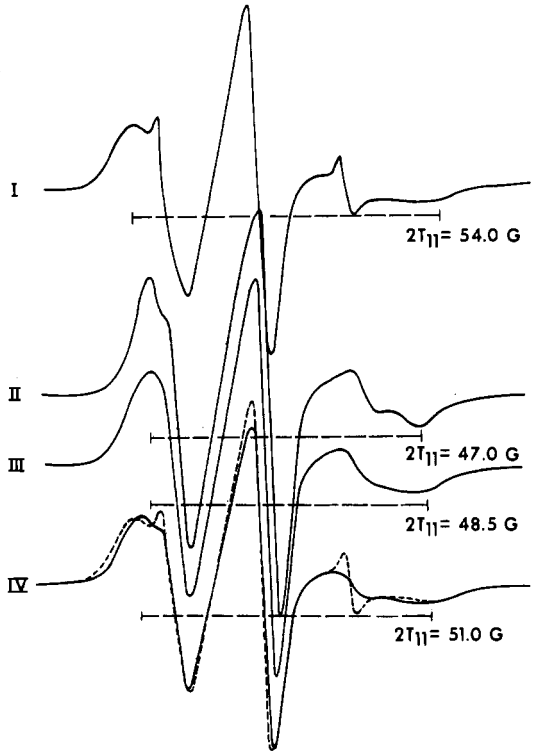


FIGURE 9: Reversibility of components in the ISL-SR spectrum. (I) Fast labeling component as given in Figure 3, I. (II) Spectrum I after washing and resuspension, (III) after addition of 5 mM AMP-PCP and 2.5 mM EGTA, and (IV) after further addition of 5 mM $CaCl_2$; the dashed line gives spectrum I for comparison. The medium was 20 mM Mops (pH 6.8), 80 mM KCl, and 5 mM $MgCl_2$, 25 °C.

mation exhibiting two types of sites, half of each type now being unlabeled and half labeled.

It is this redistribution that has made the stoichiometric labeling experiment necessary in that a given component cannot be isolated if a substrate analogue has been removed or allowed to decompose. It should also be noted that an active substrate, such as those shown in Figure 1b, will always generate a two-component spectrum due to enzymatic turnover. The stoichiometric labeling experiments thus demand a stable substrate analogue at saturating concentrations throughout the incubation.

Redistribution also predicts that the fast and slow labeling components, when stoichiometrically labeled, should redistribute into a two-component spectrum on washing and a second addition of substrate and activators. In Figure 9, this is shown to be the case. In Figure 9, I, the stoichiometrically labeled fast reacting component is shown. After the component is washed, the unbound spectrum returns (Figure 9, II), as predicted. When only ATP is added, the small broadening noted in Figure 1a occurs and is shown for this sample in Figure 9, III. Finally, when Ca^{2+} is again introduced, a spectrum identical with the two constrained component spectrum observed with the fully labeled enzyme appears (Figure 9, IV). It should also be noted that component A is minimal when the SR is labeled in this manner.

The experiment was then repeated with enzyme in which the slow reacting component had been stoichiometrically labeled. The same pattern was observed, and as shown in Figure 10, when Ca^{2+} and substrate were reintroduced after being washed, the two constrained component spectrum appeared.

We conclude then that the two label populations represent two forms of a highly similar enzymatic site. Only half of these sites exhibits sensitivity to phosphorylation, or conditions precursory to phosphorylation, at a given time.

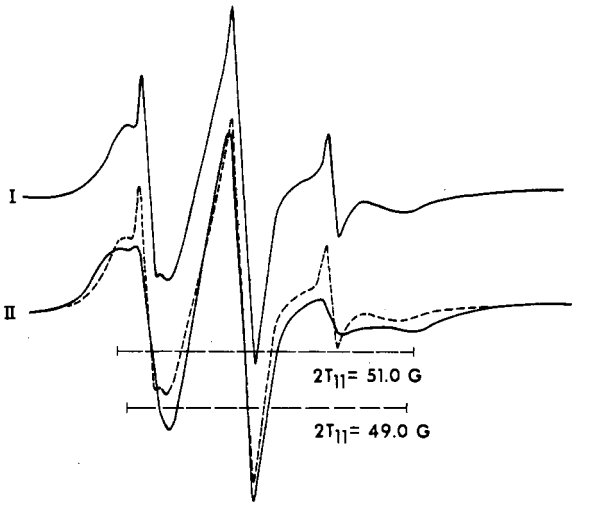
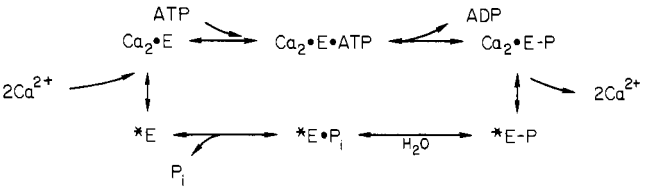


FIGURE 10: Reversibility of the slow labeling component in the ISL-SR spectrum. (I) Slow labeling component as given in Figure 3, II. (II) Spectrum I after washing and resuspension with 5 mM AMP-PCP and 5 mM $CaCl_2$. The medium was composed of 20 mM Mops (pH 6.8), 80 mM KCl, and 5 mM $MgCl_2$, 25 °C. The dashed line compares the trace and splitting parameter of spectrum I.

Scheme I



Conclusions

The phosphorylation reactions of SR ATPase with ATP or P_i may be represented with a simplified scheme of the ATPase reaction cycle (Scheme I) where E represents the enzyme state that is reactive to ATP (in the presence of Ca^{2+}) and *E is the enzyme state that reacts with P_i (in the absence of Ca^{2+}). A definite structural difference between the enzyme states in the presence and in the absence of Ca^{2+} has long been observed by spectroscopic techniques (Nakamura et al., 1972; Murphy, 1976; Dupont, 1976; Champiel et al., 1976; Ikemoto et al., 1978). Further studies indicate that these changes may be instrumental in determining specific structural interactions of the substrate with the active site (Dupont & Leigh, 1978; Inesi et al., 1980; Miki et al., 1981; Guillain et al., 1981). Our observations demonstrate that the EPR spectra of ISL-SR undergo significant changes when the catalytic site is occupied by a substrate to which the enzyme is specifically reactive: nucleotide in the presence of Ca^{2+} or P_i in the absence of Ca^{2+} and presence of Me_2SO. It is noteworthy that the occurrence of the spectral change does not require covalent phosphorylation of the aspartyl residue at the active site. This is shown by the effect of AMP-P(NH)P, which produces the spectral change even though transfer of its γ-phosphate to the aspartyl residue is impeded by the nitrogen bond, although a degree of molecular association between the γ-phosphate moiety and the residue cannot be excluded. On the other hand, the occurrence of the spectral changes does require activation of the enzyme (e.g., Ca^{2+} for the effect of nucleotides in aqueous media, Mg^{2+} for the effect of P_i in Me_2SO). These observations suggest that the spectral change observed in our experiments is related to the formation of a transition complex of the substrate with the activated enzyme, which is permissive of the phosphoryl transfer.

The specific nature of both the observed spectral change and the conformational rearrangements responsible for this change is clarified here by stoichiometric labeling experiments. The appearance of a highly constrained component on Ca^{2+} binding to the enzyme–substrate complex produces a final EPR spectrum which consists of three components, two of which can be considered to represent populations of labels in environments which constrain molecular motion and a very minor mobile component representing about 2% of the total labeled sites in our preparations. The two constrained components are not well resolved in the fully labeled enzyme spectrum and require stoichiometric labeling to facilitate separation of the spectral parameters. The major component of interest here is the third component, which only appears in the spectrum under conditions leading to active phosphorylation of the enzyme when a proper substrate is present. The enzymatic sites which constitute this component also show increased reactivity with the label, and kinetic plots give a stoichiometry of 3–4 nmol of reactive sites per mg of SR protein. This stoichiometry corresponds to the maximal levels of phosphorylation observed in our preparations.

In addition, the two components in the spectrum exhibit random redistribution when substrate is removed and then reintroduced. This was previously observed in the labeling kinetics (Coan & Keating, 1982) and indicates that the two populations of sites representing the components in the spectrum are both potentially aligned with a substrate binding site. Apparently, only half of the sites can undergo the conformational change producing the third spectral component at a given time. This spectral analysis, in terms of both isolation of components and relation to substrate sites, should prove useful in further studies of enzyme conformational organization utilizing EPR spectroscopy as well as other techniques. However, one additional point is evident here. In previous work, the specific role of the substrate in producing spectral broadening has not been clear. In part, this was because we could not differentiate between a specific conformational change in the enzyme induced by substrate binding and general restriction of molecular motion due to an interaction between the label and the bulky nucleoside moiety, such as steric hindrance, although such hindrance seems unlikely from the standpoint of the increased reactivity of the enzyme-substrate·Ca^{2+} complex toward ISL and the lack of enzyme inhibition on labeling. It should also be noted, however, that other spectroscopic techniques indicate that a conformational change is associated with enzyme phosphorylation (Miki et al., 1981; Lacapere et al., 1981; Watanabe & Inesi, 1982). Here, we see that the appearance of the third spectral component is directly related to the potential for enzyme phosphorylation, for which substrate binding is a requirement.

The specific sensitivity of the spin-label to enzyme phosphorylation is emphasized by the reaction kinetics of ISL with the ATPase, and by the effect of Me_2SO on the phosphorylated enzyme spectrum. In all cases, phosphorylation, or the close proximity of the phosphate moiety as in the case of AMP-P(NH)P, produces a marked increase in the reaction rate of half of the reactive residues. In general, the magnitude of this change follows the reactivity of the enzyme to phosphate. A further conformational change which must be sufficiently extensive to affect the motional parameters of the large nitroxide free radical is then apparent under conditions of high phosphate affinity.

In this work, we induce phosphate affinity by two very different methods, Me_2SO addition and substrate and Ca^{2+} binding at activating sites in aqueous media. For comparative purposes, it should be noted that during active turnover the steady-state phosphoenzyme that we observe (*E–P, Scheme I) does not give the fully broadened spectrum (Coan et al., 1979). This is in line with a reduced reactivity to both Ca^{2+} and ADP at this point in the enzymatic cycle, although substrate and low levels of Ca^{2+} are present in the medium. It is apparent then that the change observed in the EPR spectrum is independent of the means of activation and corresponds to a specific conformational state induced by substrate binding to the Ca^{2+}-activated enzyme in the normal mode of the cycle. These data also suggest that adenosine and Ca^{2+} may play an active role in the regulation of the enzyme by implementing the proper stereochemistry for effective utilization of the substrate. This in turn gives a more specific interpretation to the long-observed requirement for Ca^{2+} in activating the enzymatic cycle.

In addition, if the spectral change we see is related to a specific transition complex, then it would follow that the correct conformation of this complex is obtained only for an adenosine substrate. Other substrates, such as ITP and AcP (Figure 1), give intermediate degrees of spectral broadening and thus may not yield the correct conformation for effective phosphoryl transfer. These substrates are known to exhibit lower hydrolysis rates (Makinose, 1969; de Meis, 1969; Pucell & Martonosi, 1971; de Meis & deMello, 1973). It has also recently been determined that ATPβS exhibits the same overall hydrolysis rate at ATP, again pointing to a specificity for the nucleotide moiety (Pintado et al., 1982). However, in all cases, it has been established that E–P formation is very fast (Froehlich & Taylor, 1975; Verjovski-Almeida et al., 1978; Scofano et al., 1979) and would not be likely to determine the overall rate of steady-state hydrolysis even with a significant reduction in the efficiency of phosphate transfer. In accordance with this, considerable arguments have been given that the nucleotide may play a regulatory role in the rate-limiting steps of the cycle which would occur at a stage following the conversion to *E–P in the simplified reaction sequence of Scheme I (de Meis & Boyer, 1978; Verjovski-Almeida & Inesi, 1979; de Meis, 1981). For the most part, the transition of *E to E·Ca_2 has been cited as the step most likely to account for the observed nucleotide effect. However, recent work by McIntosh & Boyer (1983) gives evidence that in addition to the *E to E transition the nucleotide plays a regulatory role in both phosphoenzyme hydrolysis and the steps immediately preceding hydrolysis, concerned with the regulation of levels of the various forms of the phosphorylated enzyme intermediate. The latter is also thought to be a likely contributor to rate limitation of the cycle (Inesi et al., 1981). McIntosh and Boyer also suggest that two nucleotide binding sites contribute to regulation and that one of the sites is the catalytic site which rebinds ATP after the release of ADP. This postulation seems more in line with the conformational effects observed in the EPR spectrum. To this point, it should also be noted that recent studies, in which the rate of ATP formation in the reverse direction of the cycle was monitored, demonstrated that under controlled conditions the disassociation of ATP from the enzyme was rate limiting and consequently slower than would be expected for a diffusion-controlled, simple binding mechanism (Pickart & Jencks, 1981). This provides additional evidence that a conformationally controlled transition occurs between initial binding of the substrate and phosphoryl transfer.

In this light, the effect of Me_2SO is of additional interest. A reduction of water activity induced by additions of this solvent has previously been linked both to the high degree of

sensitivity of the enzyme to phosphate (de Meis et al., 1980) and to the unique stability of the phosphoenzyme in this solvent (de Meis et al., 1982). Here, we see that this must also include a conformational effect on the enzyme, which in turn may be influential in the stabilization of the phosphoenzyme in terms of both hydrolytic cleavage and the dephosphorylating effect produced by Ca^{2+} additions (Figure 8) which is considerably slower in Me_2SO. Such a conformational effect may also be influential in the recently observed partial phosphorylation of the enzyme by ATP in the absence of Ca^{2+} (40% Me_2SO, pH 6; Carvalho-Alves & Scofano, 1983). In fact, it appears that a conformation more similar to E–P than to *E–P (Scheme I) is stabilized in Me_2SO and that to some extent this may correspond to a nonaqueous conformation, normally induced by the nucleotide moiety, while the aqueous form, "E–P", given by the EPR spectrum in Figure 1a, is the only form obtained directly by additions of P_i in aqueous media.

Acknowledgments

I thank Dr. Giuseppe Inesi, Dr. Leopoldo de Meis, and Dr. Sergio Verjovski-Almeida for many helpful discussions throughout the preparation of this paper. I especially thank Dr. de Meis for making available titration data on the Mg^{2+} effect of enzyme phosphorylation in Me_2SO.

Registry No. ATPase, 9000-83-3; ISL, 25713-24-0; Me_2SO, 67-68-5; AMP-P(NH)P, 32476-54-3; AMP-PCP, 3469-78-1; ATP, 56-65-5; ADP, 58-64-0; ITP, 132-06-9; AcP, 590-54-5; P_i, 14265-44-2; Ca, 7440-70-2.

References

Bastide, F., Meissner, G., Fleischer, S., & Post, R. L. (1973) *J. Biol. Chem. 248*, 8385.

Berliner, L. J. (1976) *Spin Labeling. Theory and Applications*, Academic Press, New York.

Carvalho-Alves, P. C., & Scofano, H. M. (1983) *J. Biol. Chem. 258*, 3134.

Chaloub, R. M., Guimaraes-Motta, H., Verjovski-Almeida, S., de Meis, L., & Inesi, G. (1979) *J. Biol. Chem. 254*, 9464.

Champeil, P., Bastide, F., Taupin, C., & Gary-Bobo, C. M. (1976) *FEBS Lett. 63*, 270.

Champeil, P., Buschlen-Boucly, S., Bastide, F., & Gary-Bomo, C. (1978) *J. Biol. Chem. 253*, 1187.

Coan, C., & Inesi, G. (1977) *J. Biol. Chem. 252*, 3044.

Coan, C., & Keating, S. (1982) *Biochemistry 21*, 3214.

Coan, C., Verjovski-Almeida, S., & Inesi, G. (1979) *J. Biol. Chem. 254*, 2968.

Deal, W. J., Mohlam, S. G., & Sprang, M. L. (1971) *Science (Washington, D.C.) 171*, 1147.

Degani, C., & Boyer, P. (1973) *J. Biol. Chem. 248*, 8222.

de Meis, L. (1969) *J. Biol. Chem. 244*, 3733.

de Meis, L. (1981) *The Sarcoplasmic Reticulum*, Wiley, New York.

de Meis, L., & deMello, M. C. F. (1973) *J. Biol. Chem. 248*, 3691.

de Meis, L., & Boyer, P. D. (1978) *J. Biol. Chem. 253*, 1556.

de Meis, L., Vianna, A. L. (1979) *Annu. Rev. Biochem. 48*, 275.

de Meis, L., Martins, O. B., & Alves, E. W. (1980) *Biochemistry 19*, 4252.

de Meis, L., deSouza-Otero, A., Martins, O. B., Alves, E. W., Inesi, G., & Nakamoto, R. (1982) *J. Biol. Chem. 257*, 4993.

Dupont, Y. (1976) *Biochem. Biophys. Res. Commun. 71*, 544.

Dupont, Y., & Leigh, J. B. (1978) *Nature (London) 273*, 396.

Eletr, S., & Inesi, G. (1972) *Biochim. Biophys. Acta 282*, 174.

Friedman, Z., & Makinose, M. (1970) *FEBS Lett. 11*, 69.

Froehlich, J., & Taylor, E. (1975) *J. Biol. Chem. 250*, 2013.

Griffith, O. H., Dehlinger, P. J., & Van, S. P. (1974) *J. Membr. Biol. 15*, 159.

Grill, H., Weigel, N., Gaffney, B. J., & Roseman, S. (1982) *J. Biol. Chem. 257*, 14510.

Guillain, F., Champeil, P., Lacapere, J., & Gingold, M. P. (1981) *J. Biol. Chem. 256*, 6140.

Hasselbach, W. (1978) *Biochim. Biophys. Acta 515*, 23.

Hidalgo, C., & Thomas, D. D. (1977) *Biochem. Biophys. Res. Commun. 78*, 1175.

Ikemoto, N., Morgan, J. F., & Yamada, S. (1978) *J. Biol. Chem. 253*, 8027.

Inesi, G., & Landgraf, W. (1970) *J. Bioenerg. 1*, 355.

Inesi, G., Maring, E., Murphy, A., & MacFarland, B. (1970) *Arch. Biochem. Biophys. 138*, 285.

Inesi, G., Kurzmack, M., Coan, C., & Lewis, D. (1980a) *J. Biol. Chem. 255*, 3025.

Inesi, G., Kurzmack, M., Nakamoto, R., de Meis, L., & Bernhard, S. N. (1980b) *J. Biol. Chem. 255*, 6040.

Inesi, G., Kurzmack, H., Kosck-Kosicka, L. O., Scofano, H., & Guimaraes-Motta, H. (1981) Symposium on Transport and Electrochemical Coupling Muscle Contraction, Heildelberg, West Germany.

Jost, P. C., & Griffith, O. H. (1978) *Methods Enzymol. 49*, 369.

Jost, P., Waggoner, A. S., & Griffith, O. H. (1971) in *The Structure and Function of Biological Membranes* (Rothfield, L., Ed.) p 83, Academic Press, New York.

Lacapere, J., Gingold, M. P., Champeil, P., & Guillain, F. (1981) *J. Biol. Chem. 256*, 2302.

Landgraf, W., & Inesi, G. (1969) *Arch. Biochem. Biophys. 130*, 111.

Lowry, O., Rosenbrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265.

Makinose, M. (1969) *Eur. J. Biochem. 10*, 74.

Marsh, D. (1981) in *Membrane Spectroscopy* (Grell, E., Ed.) p 51, Springer-Verlag, New York.

Masuda, H., & de Meis, L. (1973) *Biochemistry 12*, 4581.

McConnell, H. M., & MacFarland, B. G. (1970) *Q. Rev. Biophys. 3*, 91.

McIntosh, D. B., & Berman, M. C. (1978) *J. Biol. Chem. 253*, 5140.

McIntosh, D. B., & Boyer, P. D. (1983) *Biochemistry 22*, 2867.

Miki, K., Scott, T. L., & Ikemoto, N. (1981) *J. Biol. Chem. 256*, 9382.

Morrisett, J. D. (1976) in *Spin Labeling. Theory and Applications* (Berliner, L. J., Ed.) p 273, Academic Press, New York.

Murphy, A. J. (1976) *Biochemistry 15*, 4492.

Nakamura, H., Hori, H., & Mitsui, T. (1982) *J. Biochem. (Tokyo) 72*, 635.

Pickart, C. M., & Jencks, W. P. (1982) *J. Biol. Chem. 257*, 5319.

Pintado, E., Scarpa, A., & Cohn, M. (1982) *J. Biol. Chem. 257*, 11346.

Pucell, A., & Martonosi, A. (1971) *J. Biol. Chem. 246*, 3389.

Scofano, H. M., Vieyra, A., & de Meis, L. (1979) *J. Biol. Chem. 254*, 10227.

Seelig, J. (1970) *J. Am. Chem. Soc. 92*, 3882.

Seelig, J., & Hasselbach, W. (1971) *J. Biochem. (Tokyo) 21*, 17.

Stryer, L., & Griffith, O. H. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 1785.

Taylor, J. S. (1981) *J. Biol. Chem. 256*, 9793.

The, R., & Hasselbach, W. (1977) *Eur. J. Biochem. 24*, 611.
Thomas, D. D. (1978) *Biophys. J. 24*, 439.
Thomas, D. D., & Hidalgo, C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5488.
Verjovski-Almeida, S. (1981) *J. Biol. Chem. 256*, 2662.
Verjovski-Almeida, S., & Inesi, G. (1979) *J. Biol. Chem. 254*, 18.
Verjovski-Almeida, S., Kurzmack, M., & Inesi, G. (1978) *Biochemistry 17*, 5006.
Watanabe, T., & Inesi, G. (1982) *Biochemistry 21*, 3254.
Watanabe, T., Lewis, D., Nakamoto, R., Kurzmack, M., Fronticelli, C., & Inesi, G. (1981) *Biochemistry 20*, 6617.
Yamamoto, T., & Tonomura, Y. (1967) *J. Biochem. (Tokyo) 62*, 558.

Polymerization of Actin and Actin-like Systems: Evaluation of the Time Course of Polymerization in Relation to the Mechanism†

Carl Frieden* and Dean W. Goddette

ABSTRACT: The time course of protein polymerization of the nucleation–elongation type is examined by using a general computer-simulation solution. For a simple nucleation–elongation scheme, it is shown that the half-time of polymerization is not necessarily a good measure of the nucleus size as has been previously suggested [Oosawa, F., & Kasai, M. (1962) *J. Mol. Biol. 4*, 10–21] since, depending on the mechanism, the apparent nucleus size, measured by a ratio of half-times at two actin concentrations, may be either larger or smaller than the real size. Steady-state equations developed by Wegner and Engel [Wegner, A., & Engel, J. (1975) *Biophys. Chem. 3*, 215–225] present a good description of the time course of polymerization although they are somewhat inflexible with regard to allowing for different mechanisms. Some of the assumptions implicit in the development of these equations are discussed in terms of the effect of changing individual rate constants or dissociation constants on the time course of polymerization. In addition, these steady-state equations have been expanded to include the consequences of a reversible first-order conformational change prior to polymerization. It is shown that a conformational change as a prerequisite to polymerization lengthens the lag time of polymerization and, depending on the conditions, may slow the rate of polymerization. The question of fragmentation and of reannealling is examined, and it is noted that simple relationships to describe these processes may not be possible. It is also shown that studies of the mechanism of polymerization require that a wide range of protein concentrations be used since data over a 2-fold concentration range can easily be fit by at least two different mechanisms. Experimental data for the polymerization of actin which demonstrate some of these points are given.

The mechanism of polymerization of actin or similar proteins has been the subject of considerable interest for a number of years (Oosawa & Kasai, 1962; Oosawa & Arakura, 1975; Korn, 1982; Wegner & Engel, 1975; Arisaka et al., 1975; Pollard & Craig, 1982). It is now generally agreed that actin and some other proteins (i.e., tubulin, bacterial flagellin) polymerize by a nucleation–elongation process in which the formation of a seed nucleus is an unfavorable process, but elongation, by monomer addition to this nucleus, is highly cooperative. While this is the overall mechanism, the specific details for the process of polymerization remain unclear. However, the specific mechanism for this type of process is of considerable interest. For example, the recognition that actin is present in nonmuscle cells as well as muscle cells has intensified interest in the polymerization–depolymerization reaction since it is believed that the process may be important in various cellular functions as well as defining the nature of the cytoskeletal structure or membrane–cytoskeletal interactions (Clark & Spudich, 1977).

General equations (Oosawa & Kasai, 1962; Wagner & Engel, 1975) have been derived for describing nucleation–elongation processes. More recently, we have developed a generalized kinetic simulation system (Barshop et al., 1983) which solves differential equations by numerical integration. We show here that this system can be used to stimulate the full time course of polymerization. Using this system, we will examine the validity of the derived equations developed previously and extend the treatment of the full time course of polymerization to include other factors as well as to questions relating to rate-limiting steps. We conclude that computer simulation of the full time course is a more satisfactory method of relating the kinetic properties of a polymerizing system to possible mechanisms.

Central to relating kinetic properties of polymerization to the mechanism is the ability to quantitatively measure the incorporation of monomer into polymer. For actin, such measurements are best carried out by using fluorescent probes which do not interfere with the polymerization process (Tellam & Frieden, 1982; Cooper et al., 1983b; Tait & Frieden, 1982a; Detmers et al., 1981). Light-scattering methods have also been used (Wegner, 1982; Wegner & Savko, 1982), but these may not be as sensitive. For tubulin incorporation into microtubules, many investigators use the change in absorbance due to turbidity. However, this method may not be a quantitative measure of polymerization since there may be a variety of macromolecular structures formed which scatter light differently (Correia & Williams, 1983). For this reason, the relation of the kinetics of polymerization to the mechanism is best examined by using the polymerization of actin. Ex-

† From the Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110. *Received May 20, 1983.* Supported by National Institutes of Health Grant AM 13332.

0006-2960/83/0422-5836$01.50/0