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Reactivity of Sarcoplasmic Reticulum Adenosinetriphosphatase with Iodoacetamide Spin-Label: Evidence for Two Conformational States of the Substrate Binding Site†

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ABSTRACT: The labeling kinetics of sarcoplasmic reticulum ATPase with the iodoacetamide spin probe *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide were followed under conditions designed to selectively label all reactive groups. Approximately 1 mol of spin-label reacted per one 100 000-dalton ATPase chain, indicating only one residue on the enzyme had been labeled. One uniform rate of labeling was observed in the presence of Ca^{2+}. When substrate was then added, approximately one-half of the residues showed a 10-fold increase in labeling rate while the remaining residues reacted at the initial, slower rate. Sequential labeling experiments further established that the two labeling rates correspond to the coexistence of two conformational states of the enzyme. Both Ca^{2+} and substrate are required to obtain an equal distribution between states, and the effect is completely reversed when substrate is removed. The iodoacetamide spin probe is known to be highly sensitive to the conformation of the ATPase binding pocket, and the residue labeled here is the one which generates broadening in the electron paramagnetic resonance spectrum on substrate binding. Due to the unique selectivity of the labeling reaction, it is suggested that when both substrate and Ca^{2+} are bound to the enzyme, conditions which are precursory to enzyme phosphorylation, two specific conformations of the binding pocket exist in approximately a 50:50 ratio.

It has been established for some time that sarcoplasmic reticulum (SR)[1] ATPase can be selectively labeled with the iodoacetamide spin probe ISL without loss of activity (Landgraf & Inesi, 1969). The spectrum of the labeled enzyme exhibits a high degree of sensitivity to substrate binding and is, in turn, regulated by the presence of Ca^{2+} (Inesi & Landgraf, 1970; Coan & Inesi, 1977).

Since the labeled enzyme remains fully functional, this technique has produced an independent means of monitoring the allosteric effect of Ca^{2+} on the substrate binding site. This has aided the interpretation of Ca^{2+} binding studies involving high-affinity sites which are precursory to enzyme activation (Inesi et al., 1980), and it has also allowed Ca^{2+} regulation of the substrate site to be followed directly during active turnover (Coan et al., 1979).

The continued use of the iodoacetamide label has made it increasingly important to ascertain the number and nature of the labeled residues on the ATPase. Kinetic labeling studies

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[1] Abbreviations: SR, sarcoplasmic reticulum; ISL, *N*-(1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide; Mops, 4-morpholinepropanesulfonate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*-*N*′,*N*′-tetraacetate; AMP-P(NH)P, adenyl-5′-yl imidodiphosphate; [SnoPP(NH)P]$_2$, disulfide of thioinosine 5′-(β,γ-methylenetriphosphate); NaDodSO$_4$, sodium dodecyl sulfate.

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provide a means of stoichiometrically determining how many residues will label and of categorizing residues according to their reaction rates. Here we follow the reaction rate of ISL with SR by monitoring the decrease in signal of unbound ISL as the reaction progresses in the sample cell of the EPR spectrometer. This technique has previously been used with success on SR (Tonomura & Morales, 1974; Coan & Inesi, 1977; Champeil et al., 1978) and relies on the difference in magnitude between the isotropic, free label signal and the greatly broadened spin-labeled enzyme signal (approximately 500:1). This in turn allows the free signal to be monitored at amplitudes where no significant contribution from labeled protein is expected.

Kinetic labeling studies may also produce new information about conformational states of an enzyme. It has previously been demonstrated that the initial labeling rates of ISL on SR ATPase are affected by the presence of substrate and Ca^{2+}, indicating that associated conformational rearrangements alter the labeling rates as well as the resulting EPR spectrum of enzyme (Champeil et al., 1978). In this study, we follow the labeling reaction to completion, using kinetic analysis to differentiate between changes in rates of labeling and in the number of available sites. In doing so, we demonstrate a unique degree of specificity for the iodoacetamide spin-label, and by the use of sequential labeling techniques, we are also able to demonstrate the coexistence of two distinct conformational states directly associated with substrate binding in the presence of Ca^{2+}.

Experimental Procedures

SR vesicles were prepared from the white skeletal muscle of rabbit hind legs by using methods previously described (Eletr & Inesi, 1972). Vesicles were stored in 30% sucrose and 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 purchased from Synvar and used without further purification.

Stock solutions of ISL (10 mM in 50% ethanol) were stored at −17 °C. Standardizations of the stock solutions were performed by titration against a solution of DL-cysteine, which had previously been standardized by using 5,5′-dithiobis(2-nitrobenzoic acid). We found these solutions to vary by only a few percent from values predicted on the basis of weight, using label directly as obtained from Synvar. The SR protein concentration in terms of milligrams per milliliter was determined by the techniques of Lowry et al. (1951) with bovine serum albumin as a standard. These values are close to those obtained by the biuret method and by the Nessler method of nitrogen determination after allowing for phospholipid nitrogen (Inesi et al., 1980). The percentage of ATPase in the total protein was obtained from $NaDodSO_4$ gel electrophoresis preformed according to the method of Weber & Osborne (1969). Assuming the M_r 100 000 band to be entirely composed of ATPase units (Inesi & Scales, 1974), we determined 80% of the protein to be ATPase, with an average variation between samples of 5%. This is in good agreement with similar experiments performed as standardizations in earlier studies (Inesi et al., 1980). The molecular weight of the ATPase was taken to be 100 000 (MacLennan, 1970). The molar ratio of ISL to ATPase could be calculated by using these values.

Labeling Experiments. All measurements were made with a JOEL-MI-IX electron spin resonance spectrometer equipped with a variable temperature accessory. Before each experiment, an 8×10^{-1} mM solution of ISL in 20 mM Mops and 80 mM KCl, pH 6.8, was prepared from the ISL stock solution. So that a standard EPR signal for unbound ISL could be obtained under conditions identical with those of the kinetic measurements, one-half of this solution was combined with SR which has been previously reacted with an equivalent amount of iodoacetamide (not spin-labeled) for 1 h at 25 °C. The other half of the 8×10^{-1} mM solution was then added to a solution of unreacted SR to initiate the kinetic experiment. The difference in signal of the two solutions was assumed to be due to the reaction of ISL with SR ATPase. When required, additions of cations and substrates were made to the SR solutions before the addition of ISL. Final concentrations were 4×10^{-1} or 2×10^{-1} mM ISL and 5.0 mg/mL SR ($\approx$4 4×10^{-2} mM). All solutions were brought to 25 °C before mixing and maintained at 25 °C in the spectrometer sample chamber.

Directly after the addition of ISL to the SR previously reacted with iodoacetamide, the line heights of three separate aliquots were measured in the EPR spectrometer to obtain the average error in signal measurement. The spectrometer was adjusted with the first aliquot in place, and no further adjustments were made between the aliquots of the standard solution or the final reaction mixture. The same sample cell was used and was carefully repositioned between each measurement. If this was done properly, no difference in frequency was noted on changing samples. When these techniques were used, the three measurements of the standard solution agreed to within 1%.

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 the kinetic experiments, only the low-field lines were scanned so data points could be taken easily at 1-min intervals. Eventually the entire 100-G range was scanned, and even at the completion of the reaction when maximal enzyme labeling had occurred, no difference in the percent of signal decrease could be seen between the high, center, and low field lines. This serves as a good indication that there were no contributions to the measured free signal from the labeled enzyme. The labeling reaction was assumed to be complete when no further signal decrease was apparent for an extended period of time (20 min). Over the time frame of an entire experiment, accurate measurements could be made of the initial reaction rates, yet there were minimal problems with denaturation, labeling of very slow groups (perhaps due to denturation), or signal fluctuations. Also, the standard solution showed no reaction with ISL and maintained a constant signal over this time frame.

As a further check on the accuracy of the standard sample, in several experiments a simple plot of time vs. signal decrease was extrapolated to $t = 0$. This value agreed well with standard sample values. In fact, in earlier work the zero point obtained from standard samples had not agreed well with extrapolation data, giving a difference of $\sim$5%, and had not been highly reproducible (Coan et al., 1979). The use of prelabeled SR and attention to signal reproducibility have essentially eliminated this problem. Furthermore, once it is established that there are no fast reacting groups which preclude EPR measurement, the extrapolation technique may prove superior in that all measurements can be made internally on one sample.

Sequential labeling experiments were performed by first reacting SR, to which substrate and cations had been added as required, with a solution of either ISL or plain iodoacetamide (non-spin-labeled) and then withdrawing aliquots at 2-, 4-, and 10-min intervals. These were diluted 10-fold in 20 mM Mops, pH 6.8, and 80 mM KCl, 4 °C, followed by centrifugation at 18 500 rpm for 90 min. The pellets were then re-

suspended to appropriate concentrations for use in a second set of ISL labeling experiments.

Protein Spectra. When spin-labeled enzyme spectra were to be measured, the SR was spun down from the reaction mixture in a high-speed centrifuge (18 500 rpm, 4 °C), washed 2 times by repeated centrifugations, and resuspended in buffer to a concentration of 25 mg/mL. For protein spectra, a modulation width of 1.25 G with a response of 3 s and a scan rate of 10 G/min was generally used.

Methods of Analysis. The reaction of SR with ISL is bimolecular, and this type of reaction has been treated successfully with second-order kinetics by using graphical methods of solution for other sulfhydryl reagents with SR (Murphy, 1976, 1978; Andersen & Moller, 1977; Ikemoto et al., 1978). The rate equation is given by

$$\ln ([SH]/[ISL]) = kt([ISL]^0 - [SH]^0) + \ln ([SH]^0/[ISL]^0)$$

where $[SH]^0$ and $[ISL]^0$ denote initial concentrations of SH and ISL, respectively. Generally the concentration of reagent is kept very high, and this becomes a "pseudo-first-order" reaction. In these experiments, however, we take into consideration the change in concentration of ISL throughout the reaction, although it remains in sufficient excess for the reaction to go readily to completion. This presents no experimental problems since [ISL] is measured directly. The sulfhydryl concentration is given by $[SH] = [SH]^0 - ([ISL]^0 - [ISL])$, where $[SH]^0$ is determined from the end point of the reaction.

When more than one class of residues is present, then $\ln ([SH]/[ISL]) = \ln (\sum_i [SH]_i/[ISL])$ and the distinguishable classes, $[SH]_i$, may be separated by fitting the linear portion of a semilog plot. Initially the slope and intercept are determined for the slowest groups, giving respectively the apparent rate constant and the number of residues in this class. The linear extrapolation of this slope is then subtracted from the experimental curve, and the difference is plotted separately to give a kinetic plot for the remaining residues. This is repeated until all groups are defined. Clearly experimental errors compound for a complex reaction in this method. Here, however, we generally have only one class of reactive residues, and at most two.

Integration of Spectra. When required, the spectrum of the spin-labeled enzyme was carefully measured and digitized. Double integrations were then performed on a North Star Horizon microcomputer. Identical procedures were performed on a standardized solution of ISL, and the integrated areas were directly compared to obtain the number of spin-labels covalently bound to the enzyme.

Results

The addition of substrate in the presence of Ca^{2+} markedly increases the reaction rate of ISL with SR ATPase, as demonstrated in Figure 1. On the other hand, when EGTA is present (i.e., no free Ca^{2+}), substrate has little or no effect on the labeling rates as demonstrated in Figure 2. In all cases, it is apparent that the labeling reaction goes to completion in the given time frame and that the same total number of residues is labeled. It is most significant that the number of labeled residues appears to be one per 100 000-dalton ATPase unit (8 nM ISL/mg of SR).

These studies are in reasonable agreement with the noted effects of ADP with Ca^{2+} or EGTA on initial labeling rates as determined by Champeil and co-workers (Champeil et al., 1978). To eliminate complications from ADP hydrolysis by myokinase, a contaminant of SR (Coan & Inesi, 1977), we have used AMP-P(NH)P, a substrate analogue (Yount et al., 1971) which undergoes minimal hydrolysis and should maintain full enzyme saturation at concentrations used here (Taylor, 1981). In addition, we have used a higher ISL:ATPase ratio to obtain a clear leveling of the reaction rates which also appears to have eliminated contributions from very slow reacting residues (Coan et al., 1979). This choice of conditions has made the unique specificity of the label apparent. In fact, since the number of residues labeling is the same in all cases, observed differences in reaction rates on substrate addition are most likely associated with variations in the apparent rate constants of the labeling residues. Separation of these parameters, however, requires a more complete kinetic treatment of the data.

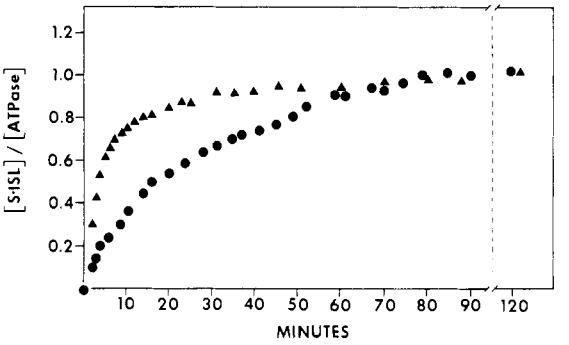


FIGURE 1: Reaction of SR ATPase with ISL in the presence of Ca^{2+}, with and without AMP-P(NH)P. Reaction conditions are 20 mM Mops, pH 6.8, 80 mM KCl, 5 mM $MgCl_2$, and 1 mM $CaCl_2$. With no further additions (●); with 5 mM AMP-P(NH)P (▲). The concentration of labeled sulfhydryl residues, [S-ISL], was calculated from the decrease in line height of the free ISL signal, as described in the text. All measurements were at 25 °C.

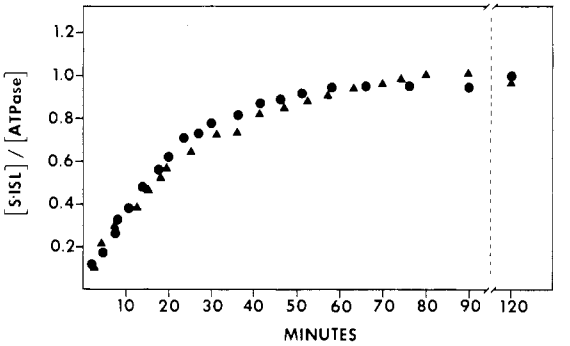


FIGURE 2: Reaction of SR ATPase with ISL in the presence of EGTA, with and without AMP-P(NH)P. Reaction conditions are the same as in Figure 1, but with 2 mM EGTA and no $CaCl_2$ (●) and with 5 mM AMP-P(NH)P (▲).

It should also be pointed out that while it has been established that ISL reacts exclusively with the SR ATPase (Champeil et al., 1978), the unique stoichiometry shown here depends on an accurate estimation of the ATPase concentration. This relies on gel electrophoresis to determine the percentage of 100 000-dalton peptide chains in the SR protein and assumes that the electrophoresis band consists entirely of ATPase units. No further separation of this band has been apparent here, or elsewhere to our knowledge. It cannot be said conclusively, however, on this basis alone, that the band consists of one unique molecular species. In this light, it is important to note that small errors in concentration could not significantly alter the ISL:ATPase ratio. In fact, the concentration would have to be in error by 100% to estimate two labels per ATPase. However, a 1:1 ISL:ATPase ratio does not necessarily mean that there is one site on each 100 000-

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

additions	no. of expt[b]	N_{slow}[c]	k_{slow}[d]	N_{fast}[c]	k_{fast}[d]	N_{total}
$CaCl_2$	12	0.96 ± 0.15	5 ± 2			0.96
AMP-P(NH)P + $CaCl_2$	18	0.43 ± 0.10	6 ± 3	0.50 ± 0.15	40 ± 20	0.93
AMP-P(NH)P + EGTA	18	0.82 ± 0.14	7 ± 3	≈0.08		0.90
EGTA	7	0.87 ± 0.11	4 ± 2	≈0.12		0.99

[a] Solutions contained the following: SR, 5.0 mg/mL; ISL, 0.4 or 0.2 mM; Mops, 20 mM, pH 6.8; KCl, 80 mM; $MgCl_2$, 5 mM; AMP-P(NH)P (where applicable), 5 mM; EGTA, 2 mM; $CaCl_2$, 10 μM, 1 mM, or 5 mM. [b] Number of experiments averaged to obtain data under given conditions. [c] Moles of ISL per mole of ATPase. [d] In (mol of ISL per mol of ATPase)$^{-1}$ min^{-1}.

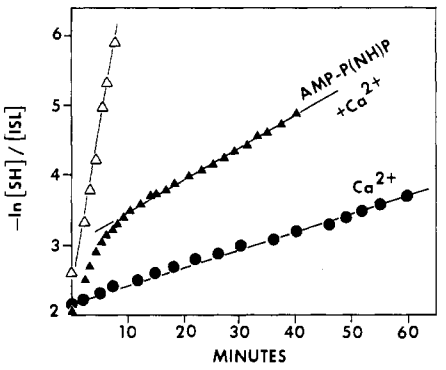


FIGURE 3: Second-order kinetic plot of the data shown in Figure 1 for the reaction of SR ATPase with ISL. In the presence of $CaCl_2$ (●) and AMP-P(NH)P + $CaCl_2$ (▲). Solid lines represent a linear regression fit to the data between t = 20 and t = 50 min. For the AMP-P(NH)P data, the difference between the experimental curve at $t \leq 10$ min and the linear fit is plotted separately (△), which in turn should give a plot of the fast reacting residues. Calculations of [SH] and [ISL] are described in detail in the text.

dalton chain. It is possible for one chain to have two sites and a very similar, but molecularly distinct, second chain to have none.

Kinetic Analysis. Once the end point of a reaction is clarified, second-order kinetic plots may be used to separate rate constants and determine the fraction of residues labeling in each group. This technique is demonstrated in Figure 3 for the effect of AMP-P(NH)P and Ca^{2+} on the labeling rates. It is clear in the figure that there are two distinct kinetic groups in the presence of substrate and only one in the presence of Ca^{2+} alone. Apparent rate constants and values for the fraction of residues in each group are given in Table I, for all conditions studied.

Several important aspects of the labeling reaction are made clear in Table I. Again, in all cases, only one SH group per 100 000-dalton chain is apparently available to label. When substrate and Ca^{2+} are both present, approximately one-half of these residues label with a 10-fold increase in rate, while the other half maintain the rate observed for all residues in the presence of Ca^{2+} alone.

Additions of EGTA made little difference to either the rates or the number of groups labeling. Further additions of substrate had little effect with EGTA present. With or without substrate, a very small fast group (approximately 0.1 residue) was apparent but was too small to characterize accurately. It should be noted, however, that this fast group would give a small increase in initial labeling rates, as seen by Champeil et al. (1978).

The data presented in Figures 1–3 were taken from one representative experiment for each set of conditions. The data in Table I, however, represent the average of many sets. Different Ca^{2+} concentrations were checked, and micromolar Ca^{2+} was sufficient to produce the substrate effect on the labeling rates. No further effects were visible at 1 or 5 mM Ca^{2+}, respectively. Several experiments were repeated by using ADP rather than AMP-P(NH)P, and the choice of substrate also had no effect over the first 20 min of the reaction, at which point the ADP was significantly hydrolyzed. Two different ISL concentrations, 5 and 10 mol of ISL/mol of ATPase, respectively, were used as an experimental control since calculated values of k and $[SH]^0$ should not depend on the ISL concentration, while both the slopes (i.e., reaction rates) and intercepts of second-order plots are very dependent on these values. Furthermore, the lower ratio allowed better resolution of fast groups while the higher ratio brought the reaction of slower residues clearly to completion under all conditions.

One of the most interesting aspects of the labeling kinetics is the equal division in labeling rates, involving what appears to be ≈0.5 residue when substrate and Ca^{2+} are both present. When a hydrolyzable substrate is used, these are the conditions which are required for enzyme phosphorylation and pump activation (Toda et al., 1978; Inesi, 1979). And, in fact, even under maximal conditions, only one-half of the ATPase chains appear to be phosphorylated at any given time in intact vesicles (Yamamoto & Tonomura, 1967; Makinose, 1969; Inesi et al., 1970). Both these experiments tend to suggest that two specific conformations of the ATPase coexist at this point in the enzymatic cycle. In both cases, however, other models cannot be ruled out. Specifically, in the case of labeling experiments it may be that there are two independent, mutually exclusive residues or that two very similar polypeptide chains exist, which are not separable in $NaDodSO_4$ gel electrophoresis but contain different SH residues. This type of situation may be resolved by the use of sequential labeling techniques.

Sequential Labeling. When independent labeling groups are present, the fast reacting groups will covalently label first, and if the reaction is quenched at intermediate points, a second labeling reaction will show higher levels of labeling of the slower groups. If the rate constants differ sufficiently, the two groups may be individually labeled in this manner.

If different labeling rates for one species of sulfhydryl groups are introduced by the coexistence of two or more enzyme conformations which are dependent on the labeling conditions, i.e., substrate binding, when the conditions are removed the enzyme should revert to the original form and the labeling residues again become indistinguishable. In a second labeling reaction, when the enzyme is resuspended after quenching and removal of reactants and substrates, the labeling groups should redistribute equally and label in proportion, according to the conditions chosen for the second reaction.

Here we have initially labeled with either ISL or plain iodoacetamide and quenched the reaction at 2-, 4-, and 10-min intervals. The SR was then resuspended after all traces of reagent and substrates were removed by centrifugation. At this point, the labeling reaction was repeated with ISL.

In the first set of experiments, AMP-P(NH)P and Ca^{2+} were added to the initial reaction medium to make both fast and slow reacting groups available. As shown in Table II,

Table II: Number of Sulfhydryl Residues Labeled with ISL after Preincubation with Reagent[a]

additions	incn time[c] (min)	N_{slow}	N_{fast}	% slow	% fast
I (Preincubation Contained AMP-P(NH)P + $CaCl_2$)[b]					
AMP-P(NH)P + $CaCl_2$	0	0.41	0.44	48	52
	2	0.41	0.34	55	45
	4	0.36	0.25	59	41
	10	0.29	0.19	60	40
$CaCl_2$	0	0.89	0.01	99	1
	2	0.58	0.02	97	3
	4	0.54	0.04	93	7
	10	0.38	0.02	95	5
II (Preincubation Contained $CaCl_2$ Only)[b]					
AMP-P(NH)P + $CaCl_2$	0	0.56	0.44	56	44
	2	0.47	0.52	47	53
	4	0.43	0.44	49	51
	10	0.31	0.39	44	56
$CaCl_2$	0	0.98	0.01	99	1
	2	0.64	0.02	97	3
	4	0.41	0.02	95	5
	10	0.38	0.01	97	3

[a] Solutions contained the following: SR, 5.0 mg/mL; iodoacetamide or ISL, 0.4 mM; Mops, 20 mM, pH 6.8; KCl, 80 mM; $MgCl_2$, 5 mM; AMP-P(NH)P, 5 mM; $CaCl_2$, 1 mM. [b] Data given under each set of conditions are an average of five complete experiments. [c] Incn time = the incubation time with either ISL or iodoacetamide, prior to a second labeling with ISL.

when the second reaction was run once again in the presence of AMP-P(NH)P and Ca^{2+}, both groups labeled in equal proportions although the total number of groups was reduced by the first labeling. This was true even for the 10-min incubation set, where the fast group if independent should be completely labeled. As would be expected, when Ca^{2+} alone was added to the second reaction medium, only one uniform group was apparent. The same results were obtained whether ISL or iodoacetamide was used in the initial incubation.

In a second set of experiments, also shown in Table II, Ca^{2+} alone was added to the initial labeling medium. In this case as well, the second labeling followed patterns identical with those of unreacted enzyme with both AMP-P(NH)P + Ca^{2+} and Ca^{2+} alone. These experiments clearly demonstrate the existence of two enzyme conformations when substrates and Ca^{2+} are present, which revert to one form on removal of substrates.

Spectral Integrations. An additional check of the unique selectivity of the iodoacetamide label was obtained by integrating the spectrum of the labeled enzyme. This technique should be independent of labeling stoichiometry and kinetics. Here all traces of free label were removed by repeated centrifugations, and the area under the absorption spectrum of the protein was compared to that of a standardized solution of free ISL; 8.0 ± 1.5 nM ISL/mg of SR (1 mol of ISL/ATPase chain) was found to be covalently bound to the enzyme by this method.

EPR Spectrum. The spectrum of ISL-labeled protein is presented in Figure 4 for plain SR (no substrate) and with AMP-P(NH)P and AMP-P(NH)P + Ca^{2+}. We found these spectra not to be dependent on the labeling conditions, provided certain precautions were taken. In the case of SR labeled in the presence of EGTA, Ca^{2+} had to be added to the medium before centrifugation to prevent enzyme denaturation and consequent alterations in the spectrum (P. Champeil, unpublished results). In this respect, there is some difference between our work and that of Champeil et al. (1978), who

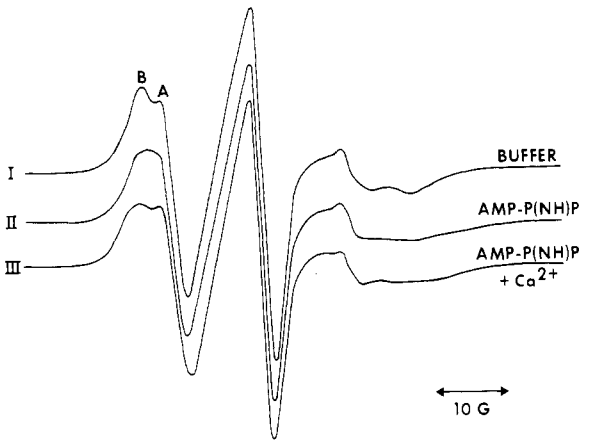


FIGURE 4: EPR spectrum of ISL·SR. (I) In buffer (20 mM Mops, pH 6.8, 80 mM KCl, and 5 mM $MgCl_2$); (II) with 5 mM AMP-P(NH)P and 1 mM EGTA; (III) with 5 mM AMP-P(NH)P and 5 mM $CaCl_2$. All spectra were measured at 25 °C.

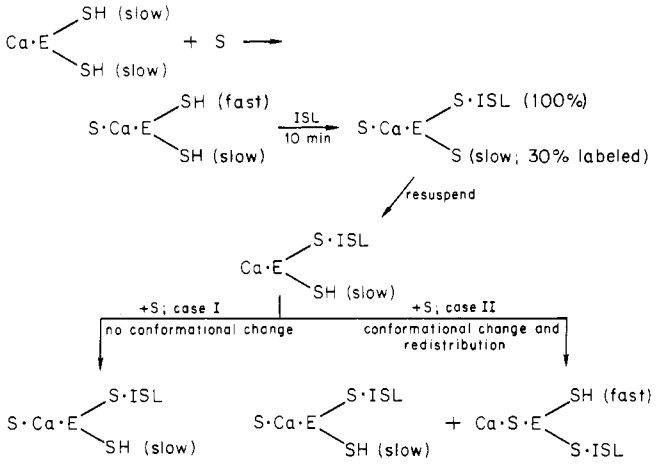
reported some spectral modification on labeling under different conditions. However, Champeil and co-workers labeled the enzyme used for spectral comparisons at higher pH, where they clearly demonstrated a higher reactivity, possibly involving more residues. This could very well be responsible for differences in the EPR spectra.

The ISL·SR spectrum has been well characterized in previous studies (Landgraf & Inesi, 1969; Coan & Inesi, 1977; Champeil et al., 1978) and can be said to mainly consist of a fairly immobile component (labeled B in the low-field lines of the spectrum), which is broadened on addition of substrate (Figure 4, II). This broadening is significantly enhanced by the addition of Ca^{2+} (Figure 4, III). A very small mobile component (A) is also discernible in the spectrum. This component is minimal in protein samples labeled in the manner described here. A significantly larger contribution is found when the labeling is carried out for longer periods of time with a much larger excess of reagent.

Enzyme labeled in the manner described in sequential experiments also produced identical ISL·SR spectra. In this case, plain iodoacetamide (not spin-labeled) was used in the initial labeling. This reaction was quenched at 2-, 4-, and 10-min intervals, and the protein spectra were taken from a second labeling with ISL. Spectra labeled in the presence of substrate and Ca^{2+} gave ISL·SR spectra identical with that in line I of Figure 4 when the substrate was washed out and showed the same degree of broadening shown in line III of Figure 4 when substrate and Ca^{2+} were reintroduced. Again since in the 10-min preincubation fast groups should have been fully labeled with plain iodoacetamide, this shows evidence of a conformationally controlled reaction. As a further precaution, SR was labeled in the presence of Ca^{2+} and substrate for 2 min with ISL (labeling primarily the fast group), and this also showed the same spectral pattern. It should be noted, however, that in spectra labeled for a very short period the highly mobile component A is virtually undetectable.

Although protein spin-labels have proven valuable as probes for conformational change, an analytical interpretation of the components in the spectrum is frequently difficult. This is usually because a given protein spectrum is a composite of many labeled residues and because there is a great variety of movement which can give mobility to a labeled residue on an enzyme surface. Considering the results of the kinetic labeling studies presented here, however, it seems plausible that the broadened spectrum consists of at least two components, representing the two distinct labeling groups present with

Scheme I



substrate and Ca^{2+}, and the spectrum of plain SR primarily one. However, this cannot be stated definitively until experiments are devised to actually separate the components in the EPR spectrum. In fact, the specific labeling obtained under the conditions used here may make the future pursuit of analytical spectral analysis more reasonable for this label.

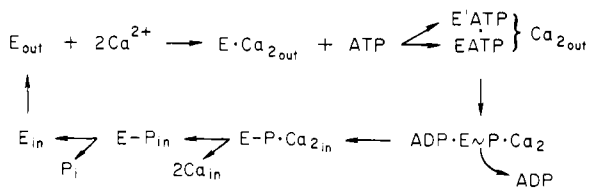
Conclusions

Primarily two conclusions can be drawn from the reaction kinetics of ISL with SR. In the first place, it is evident that the iodoacetamide spin probe is highly selective, labeling approximately one residue per SR ATPase polypeptide chain under the conditions used here. This stoichiometry is apparent from both the labeling kinetics and integration of the final ISL·ATPase spectrum. Addition of substrate and cations does not alter the specificity of the label but does affect the labeling rates.

Second, evidence is given for two conformations of the ATPase·substrate·Ca^{2+} complex, which revert to one conformation when the substrate is removed. This was initially suggested by the appearance of two specific labeling rates when both substrate and Ca^{2+} were bound to the enzyme, with a stoichiometry of approximately 0.5 residue per labeling group. A fairly conclusive test for the conformational dependence of labeling kinetics, however, is given by sequential labeling experiments. It is also important to note that the interpretations of the sequential experiments do not depend on the unique stoichiometry, also indicated here, but would be the same if two or more residues were involved. This is perhaps best seen as illustrated in Scheme I. Here fast labeling kinetics are inferred by substrate (S) to one of two labeling groups. After a 10-min incubation, the fast group should be fully labeled. In a second labeling reaction, two labeling rates require the reintroduction of a fast group. This in turn should only occur after the enzyme has reverted to an original conformation and then redistributed into two conformations when substrate is again introduced. Furthermore, if two different sulfhydryl residues are involved, then they must be interchangeable in terms of their reaction kinetics. Also, it does not appear that there is an appreciable equilibrium between the two conformations of the enzyme·substrate·Ca^{2+} complex. If an equilibrium state was present, most of the label should have reacted at the faster labeling rate, the equilibrium shifting to allow for the availability of these residues during the time course of the reaction. The equal division in labeling residues seen here should only occur when the total number of residues in each class is held constant during a given experiment.

Previous work has demonstrated a high degree of sensitivity

Scheme II



of the spin-label spectrum to the conformation of the nucleotide binding site on the ATPase (Coan et al., 1979). In fact, the spectrum shows little sensitivity if substrate is not first bound to the enzyme, and Ca^{2+} binding by itself has minimal effects at 25 °C although effects are visible at lower temperatures (Champeil et al., 1976). This high degree of specificity suggests that the conformational change we have observed here in monitoring labeling rates is also directed toward the substrate binding pocket.

We have chosen in this work to specifically look at the effect of substrate and Ca^{2+} binding to the ATPase enzyme, and we have used a substrate analogue which undergoes minimal hydrolysis to prevent phosphorylation and subsequent steps in the enzyme mechanism. This has allowed us to focus on the initial enzyme·substrate·Ca^{2+} complex, which is percursory to active transport. This may be summarized by the basic scheme initially suggested by de Meis & Carvalho (1974) and given here with some modification in Scheme II.

In this sequence, binding of Ca^{2+} and ATP to the enzyme on the membrane outer surface is immediately followed by the transfer of the ATP terminal phosphate to an aspartyl residue on the enzyme (Bastide et al., 1973; Degani & Boyer, 1973), resulting in a short-lived, high-energy, intermediate, which is followed by the rapid transfer of Ca^{2+} to the membrane interior (Froehlich & Taylor, 1975; Verjovski-Almeida et al., 1978). Ca^{2+} is then released preceding dephosphorylation (Coan et al., 1979), and the enzyme recycles. On the basis of Ca^{2+} binding studies, we also show two Ca^{2+} ions binding per active enzyme unit (Inesi et al., 1980). Although these studies indicated a binding ratio of one Ca^{2+} ion per ATPase chain, in the activated enzyme binding occurred with a ratio of two Ca^{2+} ions per site phosphorylated by ATP under equilibrium conditions, as was previously reported by Yamamoto & Tonomura (1968) and Makinose (1969). Furthermore, these binding studies, as well as those of McIntosh & Berman (1978), established a highly cooperative mechanism for Ca^{2+} binding which ultimately must involve two sites per functioning unit.

We have used E and E′ to represent the two enzyme conformations which give different reactivity to the sulfhydryl residue. The kinetic data represent the first three steps in the scheme, the first two states E and $E \cdot Ca_2$ giving uniform reaction kinetics. By the use of AMP-P(NH)P, we hoped to have held the enzyme in the tertiary binding complex, which is, of course, where we see evidence of two separate conformational states. We cannot say directly whether there is one of each kind in a two-state functioning unit, but only that the two states are interconvertible. Our scheme, partly for purposes of illustration, shows a division into single distinguishable units, which may or may not be associated, but probably only one of which can lead to phosphorylation at any given time.

On the other hand, evidence for two distinct types of ATP binding sites comes from kinetic studies of substrate utilization. It has been well established that the rate of hydrolysis of ATP is biphasic (Inesi et al., 1967; Yamamoto & Tonomura, 1967; de Meis, 1971; The & Hasselbach, 1972; de Meis & de Mello, 1973). At micromolar ATP concentrations, an initial hy-

drolysis rate occurs which will produce maximal levels of E–P (4 nM/mg). At millimolar concentrations, the rate increases; however, since maximal levels of phosphoenzyme can be produced at the initial rate, it has been suggested that this increase in rate is due to binding at a secondary activating site (Verjovski-Almeida et al., 1978; Taylor & Hattan, 1979). Furthermore, due to the unique "half-site" stoichiometry of phosphorylation, it has been suggested that the enzyme is in a two-site unit, where the activating site is an alternate form of the primary phosphorylation site (Froehlich & Taylor, 1976; Verjovski-Almeida & Inesi, 1979).

Further evidence for two specific ATP sites with different reactivities can be found in chemical modification studies. Patzelt-Wenczler et al. (1980) have demonstrated two specific rates of competitive inhibition of the ATPase with $[SnoPP-(NH)P]_2$, while Pick & Bassilian (1981) have indicated that the binding of one fluorescein isothiocyanate molecule per two ATPase chains will completely inhibit the enzyme. In addition, it has recently been reported that dicyclohexylcarbodiimide (DCC), an inhibitor of Ca^{2+} binding, requires a stoichiometry of 4 nmol of DCC/mg of protein, or one site per two ATPase chains, to inhibit ATPase activity (Murphy, 1981). The latter two studies certainly suggest an association in which modification of one of two chains fully inhibits the active enzyme unit.

While the biphasic substrate hydrolysis and the modification studies demonstrate the presence of two types of binding sites, the relationship between the sites has not yet been fully established. It is not clear whether there are two distinct, independent sites, only one-half of which may be available to react at a given time, or one site which takes on two characteristics under specific conditions. The binding stoichiometry is also not clear as to whether there is a total of two or four sites per two chains. Similarly the Ca^{2+} binding stoichiometry does not indicate the actual number of sites per chain, only that two sites function in a cooperative manner per two-chain unit.

One unique aspect of the work presented here is that it demonstrates the presence of two forms of the enzyme complex directly in terms of the enzyme conformation. These forms also appear, within the sensitivity of our labeling methods, to be fully interchangeable and to have a stoichiometry of one per ATPase chain. If our stoichiometry is correct, and we are indeed monitoring the active site conformation (Coan et al., 1979), then it would appear that one of the two conformations demonstrated here is the phosphorylating form of the enzyme, while the other represents an alternate form of this site, which may or may not be the activating site. In fact, a dimeric form of the $ATP{\cdot}E{\cdot}Ca_2$ complex very similar to that initially proposed by Verjovski-Almeida & Inesi (1979) seems to best explain our data. In this case, two ATPase chains each containing one substrate binding site would form one active enzyme complex, so that when both substrate and Ca^{2+} are bound, the conformation of one of the two sites is altered in such a manner that the terminal phosphate of ATP can be effectively transferred to the phosphorylation site. These sites then become indistinguishable to our labeling reagent on removal of substrate. Clearly a reasonable conformational similarity between the two sites must exist at this point. In any case, the high degree of interconvertibility between forms demonstrated here certainly suggests that these sites are contained on reasonably similar polypeptide chains.

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