Effect of Diethyl Pyrocarbonate Modification on the Calcium Binding Mechanism of the Sarcoplasmic Reticulum ATPase*

Carol Coan‡ and Rudene DiCarlo

From the Department of Physiology, School of Dentistry, University of the Pacific, San Francisco, California 94115

Dietethyl pyrocarbonate was used to modify histidyl residues on the sarcoplasmatic reticulum ATPase. Difference spectra of the N-carbethoxyhistidyl derivative indicated that most all the histidyl residues on the enzyme had been modified. These residues could be divided into two populations on the basis of their reaction rate with the reagent. It could then be shown that enzyme inhibition followed modification of the slower reacting population. Reversal with hydroxylamine verified that the loss of activity was due specifically to histidyl modification. Using [32P]ATP as a substrate it was further determined that the modified ATPase could form a phosphoenzyme intermediate, but that the hydrolysis of this intermediate was inhibited. Size exclusion chromatography was used to obtain equilibrium binding curves for high affinity Ca2+ sites on the enzyme. With the normal ATPase a cooperative binding curve for two Ca2+ with a Hill coefficient of 1.8 was observed. With the modified ATPase binding to two independent sites was observed; however, the dissociation constants remained the same as in the cooperative mechanism (K1 = 14 mM; K2 = 0.5 mM). That is, modification had eliminated cooperativity without changing the site specific binding affinities. E-P formation was then shown to follow binding to the higher affinity of the two sites. This would be the second site to bind Ca2+ in a sequential, cooperative mechanism. A model is suggested in which the binding of Ca2+ to an initial site allows for the binding of a second Ca2+ to an occluded site, this second site being responsible for enzyme activation. Modification apparently allows the binding properties of both sites to be observed independently.

It has been established for some time that modification of the SR ATPase by reagents which exhibit a high reactivity toward histidine causes inhibition of enzyme activity (Tenu et al., 1976a; Coffey et al., 1975; Yu et al., 1974; Martonosi et al., 1972). At the time of these studies, however, no singular step could be associated with the loss of activity, and there were conflicting reports as to whether or not the modified enzyme could be phosphorylated by ATP. It has since been demonstrated that the induction of cooperativity into the Ca2+ binding mechanism is pH-sensitive (Inesi and Hill, 1983) with an apparent pK which is similar to that normally associated with a histidyl residue. In turn, this suggested an investigation into the effect of histidine modification on the mechanistic steps which are associated with Ca2+ binding and the subsequent activation of the enzyme. Dietethyl pyrocarbonate (DEPC, 1 ethoxyformic anhydride) was chosen because the studies by Tenu and coworkers (1976a) showed a high degree of selectivity of this reagent for histidyl residues on the ATPase, and because the modification can be reversed by the addition of hydroxylamine. The reversal is exclusive for the N-carbethoxyhistidyl derivative (Melchior and Fahnrey, 1970), providing a means for discerning specifically those properties which are altered by His modification, as opposed to those associated with nonspecific labeling.

Ca2+ binding to high affinity sites on the ATPase is normally a stringent requirement for phosphorylation by ATP, and it is generally accepted that these high affinity sites are the transport sites as well. The structural model developed by MacLennan, Green, and coworkers (MacLennan et al., 1985; Brandl et al., 1986; Clarke et al., 1989) shows the Ca2+ binding sites to be on very different segments of the enzyme than either the ATP binding site or the phosphorylation site, which are themselves on different hydrophilic domains. Although it is difficult to postulate the physical relationship of these sites until the tertiary structure of the large hydrophilic domains is better understood, an estimation of the distance between the Ca2+ binding sites and the nucleotide binding site has been given as 35-46 Å (Highsmith and Murphy, 1984; Scott, 1985) and recent studies now indicate that the Ca2+ sites are actually within an intermembraneous channel (Clarke et al., 1989). Thus, the process of activation may be fairly complex and may involve significant changes in enzyme conformation. Qualitative evidence for conformational changes which accompany Ca2+ binding and the subsequent activation process have been observed with a variety of experimental techniques, however, very little is actually known about the structural nature of these changes or about their functional role in enzyme activation (for reviews see Inesi, 1985; Martonosi and Beeler, 1983). To the extent the data presented by these modification studies help to discern the connection between Ca2+ binding, the cooperativity associated with this binding, and enzyme phosphorylation, they should make a significant contribution toward understanding of important aspects of the transport mechanism.

* This work was supported by National Institutes of Health Grants GM38073 and RR05301 and by American Heart Association Grant-in-Aid 880724. The costs of publication of this article are defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept of Physiology, School of Dentistry, University of the Pacific, 2155 Webster St., San Francisco, CA 94115.

1 The abbreviations used are: DEPC, diethyl pyrocarbonate; SR, sarcoplasmic reticulum; AMP-PCP, β,γ-methylene adenosine 5′-triphosphate; ISL, iodoacetamide spin-label (N-1-oxy-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrito)]tetraacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); C12Es, 9 lauryl ether; SDS, sodium dodecyl sulfate; BCA, bicinchoninic acid.

5376
EXPERIMENTAL PROCEDURES

SR vesicles were prepared from the white skeletal muscle of rabbit hind legs by using methods previously described (Eletr and Inesi, 1972). Vesicles were stored in a buffered sucrose medium (30% sucrose, 100 mM KCl, 10 mM Tris, pH 6.8) at 4 °C and used within 5 days of preparation. ATP, AMP-PCP DEPC, p-enolpyruvate, lactic dehydrogenase, pyruvate kinase, bovine serum albumin, ionophore A23187, Reactive Red-agarose, C12E9, DTNB, and ISL (N-(1-oxo-2,2,6,6-tetramethyl-4-piperidinyl)iodoacetamide) were purchased from Sigma. [γ-32P]ATP and [32P]ATP were purchased from ICN Radioc.

Protein concentrations were measured using folin reagent according to the techniques of Lowry et al. (1951) using BCA reagent (Pierce Chemical Co.) according to the techniques of Smith et al. (1985). Bovine serum albumin was used as a standard in both procedures. Concentrations obtained with BCA reagent agreed to within 10% of those obtained with folin reagent, with the added advantage that the BCA reagent could be used in the presence of the detergent C12E9. ATPase activity was monitored using a coupled assay system with pyruvate kinase (40 μg/ml), p-enolpyruvate (1 mM), MgCl2, 100 μM OMPs, 80 mM KCl, 1 μg/ml ionophore A23187, and approximately 10 μg/ml SR. Coupling was checked by doubling the levels of enzymes. To determine steady state levels of phosphoenzyme, 0.5-ml aliquots of a DEPC-SR reaction mixture (10 μM CaCl2, 100 mM KCl, 100 mM MES, 100 mM KCl, pH 6.2) containing 0.3 μg of SR were quenched at given times by the addition of 0.5 ml of 14 mM histidine. The ATPase (specific activity 20,000 counts/nmol) was then added to give a final concentration of 25 μM and the solution was maintained at 5°C. After 15 s, 200 μl of 25% (w/v) trichloroacetic acid containing the SR vesicles were collected by vacuum filtration through prewashed filters (a glass Gelman on top of a Millipore type HAWP). The washing solution consisted of 5% (v/v) trichloroacetic acid containing 1 mM P and 1 mM MgATP. Following filtration the vesicles were washed with an additional 20 ml of this solution and the filters containing the vesicles were then submerged in 5 ml of Opti-Fluor (Packard) for measurement of retained radioactivity. Background measurements were obtained by substituting 5 mM EGTA for 5 mM CaCl2 in the reaction mixture. Following filtration and washing procedures, background counts/min were less than 10% of a typical sample.

DEPC-ATPase—To obtain DEPC-ATPase, 0.7 mg/ml vesicular SR was incubated with 7 μM DEPC at pH 6.2 (100 mM MES, 100 mM KCl) and 10 °C for 20–45 min, depending on the degree of labeling required. When the rate of loss of activity due to DEPC modification was less than 5%, the reaction was terminated by adding 5-mM EGTA to the reaction mixture at given times and added to a spectrophotometer cuvette containing 1 ml of the activity assay and the change in slope due to ATP hydrolysis was measured directly. It was assumed that the dilution in combination with 5 mM ATP quenched the DEPC-ATPase reaction. To obtain a control for the activity measurements, 7 mM histidine was added to the reaction mixture before the addition of DEPC. This was followed with 1 ml of 10 mM EGTA, diluted 3-fold with the appropriate buffer, and concentrated by centrifugation (42,000 X g, 90 min). The pellet was added to a volume of 500–200 μl. Difference spectra of the DEPC-His derivative were obtained by adding a 10-μl aliquot of a 0.7 M DEPC stock solution to 1 ml of 0.7 mg/ml SR suspension. The stock solution was prepared by diluting DEPC into ethanol immediately before use. 10 μl of ethanol was added to 1 ml of the same SR suspension in the reference cell. The rate of the DEPC-SR reaction was determined by monitoring the change in absorbance at 242 nm. With vesicles containing a slight clarification of the suspension was noted on the addition of reagent. To ensure that the proper base line had been obtained, the spectrum was scanned between 350 and 220 nm following the completion of the reaction. To account for the void time due to mixing (approximately 30 s), which was subtracted from the reaction time when the base line was used. To further ensure that the end point of the reaction agreed in magnitude with the maximum of the difference spectrum obtained in the full spectral scan, the modified proteins were solubilized in sodium dodecyl sulfate and the solution was scanned a second time. Problems associated with clarification were eliminated when the SR proteins were solubilized with C12E9 before modification.

Reversal of DEPC Modification—To cleave the His-CO2H bond, aliquots were taken from the DEPC-ATPase reaction mixture at given times and added to NH4OH (pH 7.5) which had been freshly prepared (final concentration 0.5 M NH4OH). After a 30-min incubation, an aliquot was added to the activity assay and the rate of ATP hydrolysis measured. In control experiments aliquots from the DEPC-ATPase reaction mixture were assayed directly.

Purification of SR ATPase by Affinity Chromatography—An affinity column utilizing Reactive Red-120 on an agarose matrix was prepared according to the methods of Coll and Murphy (1984). SR proteins (1–2 mg/ml) were solubilized in a buffer containing 20 mg/ml C12E9, 20% sucrose (w/v), 1 mM CaCl2, 1 mM MgCl2, and 50 mM MES, pH 6.2. SR ATPase was purified by allowing the DEPC-ATPase reaction mixture to centrifuged at 15,000 × g for 15 min to remove precipitated protein (primarily caseinaphosphatase). A column load of 12 mg of SR protein/ml of Reactive Red-agarose was used as we found this ratio to minimize the fraction left as a residue on the column without overloading the binding matrix. Nonreactive proteins and lipids were eluted by washing with the buffer used for solubilization. The ATPase was then removed by eluting with 400 μM AMP-P(NH)P. Solubilization buffer containing 9 mM NaF was used to remove remaining proteins from the column. The AMP-P(NH)P was separated from the ATPase either by dialysis or by running the AMP-P(NH)P-ATPase solution onto a second Reactive Red 120 agarose column, washing to remove AMP-P(NH)P, and eluting the ATPase with buffer containing 2 N NaCl. When measuring enzyme activity or levels of [32P]P formation with the purified ATPase, 4 mg/ml C12E9 was added to the assay media.

To determine the reactive cysteine content, the solubilized purified ATPase was titrated with DTNB according to the general method of Murphy (1976). In these experiments, a 10-μl aliquot of a DTNB stock solution in ethanol was added to an aliquot of 1 mg/ml ATPase from the affinity column in which the pH had been adjusted to 7.8, so that the final concentration of DTNB was 1 mM. The reaction was allowed to continue until no further changes in the absorbance spectrum were observed (at 25°C, approximately 15 min). The number of labeled cysteine residues was calculated using t = 13.6 mM−1 cm−1. To determine the reactive lysine content, the solubilized, purified ATPase was titrated with fluorescamine following a modification of the procedure devised by Hidalgo and coworkers to label SR vesicles (Hidalgo et al., 1982). As fractions of purified enzyme were used, there was no need to be concerned with the labeling of aminophospholipids. To initiate the reaction, 10 μl of a 20 mM stock solution of fluorescamine in acetone was added to a 1 ml/gm aliquot of purified ATPase which had been adjusted to pH 0.5 with solid sodium borate. Under these conditions the reaction is known to be completed in seconds (Hidalgo et al., 1982). 25 μl of the reaction mixture was then added to 2 ml of buffer and the fluorescence intensity was measured with excitation and emission wavelengths of 355 nm and 455 nm, respectively. The process was repeated with the ATPase which had been previously modified with DEPC, both in vesicular and the purified form, and the relative intensities were compared.

Ca2+ Binding—Size exclusion chromatography (Bio-Gel P-10, 50–100 mesh) was used to determine the degree of saturation of DEPC-modified SR and normal SR with Ca2+. According to procedures given in detail in Inesi et al. (1980). The protein concentration was determined individually for each column sample and calculated values for Ca2+ bound per mg SR were averaged for the set of samples obtained from each column eluent. Ca2+/EGTA buffers, adjusted to pH 6.5, were used to maintain a constant free Ca2+ concentration on the column. Before use, the DEPC-ATPase stock solution was dialyzed by titration at pH 9.5 with CaCl2, atomic standards using Arsenazo III as an indicator. At pH 6.8 free Ca2+ concentrations were estimated with the aid of a computer program using constants obtained from Martell and Smith (1974) and taking into account the competitive effects of Mg2+ and K+. (Fabiato and Fabiato, 1979).

Downloaded from http://www.jbc.org/ by guest on October 20, 2017

5377

DEPC Modification of the SR ATPase
by modification with DEPC. To prepare samples for EPR measurements, aliquots of concentrated solutions of CaCl₂, MgCl₂, EGTA, and AMP-PCP were added, as required, to 40 mg/ml SR to give a final volume of approximately 50 μl. For Ca²⁺ titrations aliquots were taken from the standardized EGTA/Ca²⁺ stock solutions so that the final concentration was approximately 10 mM with respect to EGTA.

An EM300D spectrometer (X band) interfaced with an IBM S-9001 computer and with an ER-4111VT variable temperature control system was used with IBM EPR apparatus software for all experimental measurements and data analysis. A field modulation of 100 KHz, a microwave power of 19.9 milliwatts, a modulation amplitude of 2.5 G, and a scan rate of 100 G/500 s was used in all cases.

RESULTS

Modification of histidyl residues by DEPC results in the formation of a N-carbethoxyhistidyl derivative which is characterized by an absorbance maximum at 242 nm (ε = 3.2 M⁻¹ cm⁻¹), allowing the modification reaction to be followed spectrophotometrically. Difference spectra between vesicular suspensions of modified and normal SR (Fig. 1) indicate that approximately 85 ± 10 nmol of DEPC reacts with 1 mg of SR protein (an average of six preparations, measured directly on the vesicular suspension and following SDS solubilization). The solution contained 0.7 mM DEPC, 1 mM CaCl₂, 100 mM KCl, 100 mM MES, pH 6.2, 10°C. The solid base line in b gives the spectrum after a second incubation with 0.5 M hydroxylamine.

Fig. 1. Difference spectra of DEPC-SR. a, change in absorbance at 242 nm following the addition of DEPC to vesicular SR. b, full spectrum after a 40 min incubation. The solution contained 0.7 mg/ml SR, 7 mM DEPC, 1 mM CaCl₂, 100 mM KCl, 100 mM MES, pH 6.2, 10°C. The solid base line in b gives the spectrum after a second incubation with 0.5 M hydroxylamine.

Although 95% of the total ATPase activity and virtually all of the Ca²⁺-dependent ATPase activity can be attributed to the SR ATPase in our preparations (Inesi et al., 1976), other proteins are associated with the vesicles and these may also react with DEPC. For this reason the ATPase was solubilized in the nonionic detergent C₁₂E₉ and isolated by affinity chromatography using a Reactive Red-120-agarose column. A typical elution profile is shown in Fig. 2a. In fractions where protein was indicated by a change in optical density at 280 nm, the modulus amplitude of 2.5 G, and a scan rate of 100 G/500 s was used in all cases.

Fig. 2. Elution profile of solubilized SR proteins from a Reactive Red-120-agarose column. a, DEPC was incubated with individual fractions following elution from the column. b, DEPC was incubated with vesicular SR before solubilization. AMP-P(NH)P (100 μM) and NaCl (2.0 M) were added where indicated. The profile, given by the absorbance at 280 nm, is compared to the protein concentration (Δ), to the ATPase activity (O), and to the DEPC-His content of the given fractions (■). The length of the bar indicates the two fractions which were combined to obtain sufficient protein for a difference spectrum (fraction size = 0.5 ml). The average activity for the fractions which constituted the major ATPase peak in a was 11 μmol/min/mg ATPase. The absorbance of 1 mg of ATPase is approximately 1.0 absorbance unit (ε₂₈₀ = 1.03 for intact SR; Thorley-Lawson and Green, 1973).

In the profile shown in Fig. 2a, 67% of the protein added to the column (approximately 15% of the total SR protein, mostly casein, precipitates on solubilization; see Experimental Procedures), 68% of the His which could be modified by DEPC was then determined by monitoring changes in the difference spectrum at 242 nm on addition of the reagent (solid bars).

In the profile shown in Fig. 2a, 67% of the protein added to the column (approximately 15% of the total SR protein, mostly casein, precipitates on solubilization; see Experimental Procedures), 68% of the His which could be modified by DEPC, and 90% of the ATPase activity was found in the fractions eluted by AMP-P(NH)P. As was observed by Coll and Murphy (1984), these fractions gave a consistent specific activity which was close to twice that of the solubilized SR before purification (11 μmol/min/mg versus 6 μmol/min/mg/ml at 25°C) and SDS-slab gel electrophoresis showed evidence of only the 110,000-Da ATPase protein. From three preparations, including the one depicted in Fig. 2a, an average of 106 ± 5 nmol of DEPC- His/mg ATPase was obtained. Assuming that these fractions consisted solely of the 110,000-Da ATPase, this ratio would predict that approximately 11 His/ATPase chain were reactive with DEPC. The primary sequence includes 11 His (MacLennan et al., 1985), and although some variation in the absorbance of a given protein residue may be expected from environmental factors, it is evident that most all His are reactive when the enzyme is solubilized in C₁₂E₉.

The fractions which followed the solvent front contained small amounts of nonspecific proteins (8% of the total protein in Fig. 2a) and solubilized phospholipids which produced an increase in optical density due to turbidity. If the column load concentration was measured using BCA reagent (open triangles in Fig. 2) and the presence of ATPase was determined by an activity assay (solid circles). The number of histidyl residues which could be modified by DEPC was then determined by monitoring changes in the difference spectrum at 242 nm on addition of the reagent (solid bars).
was properly adjusted, we found 1% or less of the ATPase activity in these fractions. The proteins which were present contained approximately 7% of the total DEPC-His. The third peak, which consisted of fractions eluted with 2 M NaCl, contained traces of ATPase which were the residual of the binding equilibrium between the enzyme and AMP-P(NH)P. From the level of activity the ATPase was estimated to be 8% of the total. These fractions have been shown to contain Ca²⁺ binding proteins and additional minor proteins (Coll and Murphy, 1984) which, in this preparation, constituted 17% of the total protein and contained 17% of the total DEPC-His. No further analysis was made on this fraction as the major objective of the separation was to obtain the fraction of purified ATPase.

The degree of His modification of the proteins in the intact vesicles was then determined by first reacting the SR with DEPC and then solubilizing and purifying the ATPase. An elution profile is given in Fig. 2b, and it is evident that when modified by DEPC the ATPase will no longer bind to the Reactive Red-120-agarose matrix. The fractions following the solvent front contained 74% of the total protein and 75% of the DEPC-His. SDS slab gel electrophoresis showed evidence of the 110,000-Da ATPase protein, with barely detectable contribution from minor proteins. Measurements of ATPase activity showed that 95% of the enzyme had been initially inhibited by the modification. The small fraction eluted with AMP-P(NH)P contained 6% of the protein and accounted for the remaining activity. The fractions that eluted with 2 M NaCl showed no ATPase activity and contained 24% of the protein and 25% of the DEPC-His. Out of a total of four preparations, an average of 105 ± 12 nmol of DEPC-His/mg protein was observed in the fractions following the solvent front. The small amount of non-ATPase protein normally observed in this fraction (Fig. 2a) would account for no more than 10 nmol/mg, and probably less. This would predict that approximately 10 His/ATPase, or almost all of the His in the vesicular ATPase, had been modified by the reagent.

With both the purified ATPase and the vesicular SR, the modified residues can be divided into two populations on the basis of their reaction rates with DEPC. As is shown in Fig. 1b, the DEPC reaction is characterized by a steep rise in initial slope, followed by a slow increase which levels off after 30-40 min. When the data is presented in the form of a pseudo first order plot the two populations can be resolved (Figs. 3 and 4). With the purified ATPase (Fig. 3) the y axis intercept obtained by extrapolation indicates that five DEPC-His (48 nmol/mg) are in the slow reacting population, leaving six in the fast reacting population (68 nmol/mg). A separate plot of the fast reacting population can then be obtained by subtracting extrapolated values for the slow population from the measured curve (V in Fig. 3). This plot exhibits proper linear behavior and yields a rate constant which is approximately an order of magnitude greater than that of the slow population (kᵢ = 0.076 M⁻¹ min⁻¹ versus kₛ = 0.010 M⁻¹ min⁻¹). Although the vesicular SR contains proteins other than the ATPase, both the rates of reaction (kᵢ = 0.082 M⁻¹ min⁻¹ and kₛ = 0.010 M⁻¹ min⁻¹) and the relative numbers of His in the fast and slow reacting populations (46 and 39 nmol/mg, respectively) are similar to those found with the purified enzyme (Fig. 4), and while the percentage of ATPase His in each population cannot be independently determined it is clear that contributions of non-ATPase DEPC-His do not significantly affect the overall pattern of modification. The reaction medium of both the solubilized ATPase and the vesicular SR contained 1 M Ca²⁺. When EGTA replaced CaCl₂ in the vesicular medium neither the number nor the reaction rates of the given populations changed within experimental error. When solubilized in C₂E₉ the ATPase is not stable if Ca²⁺ is removed from the medium (Moller et al., 1980; Kosk-Kosicka et al., 1981).

Although DEPC is highly specific for histidine, under certain conditions (e.g. pH 7) the reagent has been reported to react at low levels with lysine, cysteine, and tyrosine (Miles, 1977). However, DEPC modification eliminates the tyrosine contribution to the absorbance spectrum at 280 nm (Miles, 1977; Melchior and Fahrney, 1970). No alterations in the ATPase spectrum were observed in this region. In general DEPC shows minimal labeling of cysteine below pH 7 as the thiol anion is the more reactive species. However, the DEPC-Cys derivative has an absorbance maximum at 230 nm which does not reverse on the addition of hydroxylamine (Melchior and Fahrney, 1970). The spectrum of DEPC-ATPase shows no evidence of this derivative (Fig. 1b). In addition, the free Cys content of the solubilized ATPase was determined by titration with DTT (Murphy, 1976) and no difference was observed when the enzyme was first modified by DEPC. Tenu and coworkers (1976a) synthesized [¹⁴C]DEPC and obtained an amino acid analysis on labeled SR. They found no evidence of Tyr or Cys modification by DEPC, but did report labeling of approximately 10 nmol of DEPC-Lys/mg SR protein.

---

**Fig. 3.** Rate of histidine modification compared to the loss of activity of purified ATPase. The change in absorbance, A, at 242 nm is given in the form of a pseudo first order plot where A'' is determined from the end point of the reaction (approximately 50 min, see Fig. 1). The line through the data points gives the least squares fit to the slow reacting population. Δ gives points for the fast reacting population obtained by subtracting the data points from the extrapolated line. Δ compares the loss in ATPase activity, where act', the activity before the addition of DEPC, is 11 μmol/mg/min. Rate constants calculated from the slopes are given in the text.

**Fig. 4.** Rate of histidine modification compared to the loss of functional properties of the ATPase in vesicular SR. Data are given in the form of pseudo first order plots. ● gives the absorbance at 222 nm, ○ compares the loss in ATPase activity, and △ compares the loss in phosphoenzyme formation. A" was taken from the end point of the reaction, act" was 4 μmol/mg/min, and E-P" was 3.5 nmol/mg. A second measurement of act/act" following incubation with hydroxylamine is given by × for the selected points indicated by the arrows. A separate fit for the fast reacting population was very similar to that given in Fig. 3 and is not shown here. All lines give least squares fits to the respective data points. Rate constants calculated from the slopes are given in the text. Conditions of measurements are given under "Experimental Procedures."
These Lys were very fast reacting and did not affect ATPase activity. We used fluorescamine to titrate Lys residues on the solubilized, purified ATPase. Fluorescamine is known to be highly selective for lysine and has been shown to rapidly modify 75% of the Lys residues on the ATPase, with the remaining 25% not available to the solvent (Hidalgo and Ikemoto, 1977). Again, we found no detectable difference in the degree of fluorescamine labeling when the ATPase had been prelabeled with DEPC. Therefore, it is quite possible that the nonfunctional DEPC-Lys observed by Tenu and coworkers (1976a) were on proteins other than the ATPase. It should also be noted that DEPC has been used recently to modify histidyl residues on a variety of proteins under conditions similar to those used here (Sams and Mathews, 1988; Gordillo et al., 1988; Sokol et al., 1988; Gacheru et al., 1988; Pasta et al., 1987; Bateman and Hersh, 1987; Thompson et al., 1986; Grillo and Aronson, 1986; Miyamoto et al., 1986; Abdulwajid and Wu, 1986). In all cases, no evidence of tyrosine or cysteine modification was noted, and in only two cases, modification of a single, highly reactive lysine was observed (Sams and Mathews, 1988; Pasta et al., 1987).

Of the previous investigators using DEPC to modify SR, only Tenu and coworkers (1976a) attempted to quantitate the reaction. Using spectral analysis they reported 10 nmol of His/mg protein to be labeled by DEPC concomitant with a loss in activity at a rate which was similar to the rate of the slow population observed here (see below). This labeling ratio was interpreted to be approximately one His/mol ATPase. They also reported a very slow labeling population which they could not resolve, and which we did not observe. This difference in stoichiometry constitutes the only major discrepancy with the work presented here. There are several possible explanations. In the first place, Tenu and coworkers (1976a) assumed that the ATPase constituted 80% of the SR protein on the basis of SDS-gel electrophoresis using the Weber and Osborn system. Although this procedure was well accepted at the time, it is now known not to resolve many minor, nonspecific proteins which can be detected with the Laemmli system (Coll and Murphy, 1984; Barrabin et al., 1984). Also, recently devised methods of purification, including the method of Coll and Murphy (1984) and the high performance liquid chromatography analysis used by Barrabin and coworkers (1984), estimate the ATPase to be 50–60% of the total SR protein, rather than 80%. Again, considering that calsequestrin which constitutes about 15% of the total protein, precipitates on the initial solubilization in C12 Eb ou the data also predict the ATPase to be approximately 60%. Second, Tenu and coworkers (1976a) stated that spectral measurements were obtained at 230 nm. However, the quoted extinction coefficient (3.2 mm⁻¹ cm⁻¹) corresponds to the absorption maximum at 242 nm. A measurement at 230 nm would underestimate the DEPC-His content by approximately 60% (Fig. 1). Furthermore, it appears that the fast reacting population of His was not resolved by Tenu and coworkers. We found this population extremely difficult to resolve in vesicular SR due to changes in light scattering at 242 nm produced by the addition of the organic reagent. This difficulty was eliminated when the proteins were solubilized.

**Effect of DEPC Modification on Enzyme Activity**—When the ATPase activity is monitored as a function of DEPC modification, a loss in hydrolytic activity is observed which is concomitant with the slower reacting population with both the purified enzyme and the vesicular SR (Figs. 3 and 4). This indicates that modification of one or more of the His in the slow reacting population prevents enzyme turnover, although it does not indicate which step in the cycle has been inhibited. However, it is well established that the SR ATPase forms an acid-stable phosphoenzyme intermediate at a step in the cycle which directly precedes Ca²⁺ translocation. In our SR preparations we typically find maximal phosphorylation levels of 4.0–4.5 nmol/mg protein, at least 95% of which can be attributed to the ATPase (Coll and Murphy, 1984; Oliveira et al., 1988). As is shown in Fig. 4, we found the rate of inhibition of E-P formation to be much slower than the observed DEPC modification of His residues and the concomitant loss in enzyme activity. For instance, when the reaction is close to completion (20 min) with no more than 10% of the activity remaining, 75% of the ATPase can still be phosphorylated by ATP (2.5–3.0 nmol of E-P/mg). It should also be noted that virtually all the His residues on the ATPase have been modified at this point, so that the slow loss of E-P must be due either to additional, non-His, DEPC reactions, or to a degree of enzyme denaturation. When the reaction of the DEPC-ATPase with the [32P]ATP is quenched by the addition of histidine buffer (pH 7.6, this is before the acid quench normally used to stabilize E-P) the remaining phosphoenzyme is stable for an indefinite period. As the phosphoenzyme formed with normal ATPase will decompose rapidly under these conditions, it is further evident that the decomposition of the phosphoenzyme has been inhibited by DEPC modification. A very similar result was observed following the photooxidation of His, both in terms of the slow rate of E-P inhibition and the stability of the phosphoenzyme once formed (Coffey et al., 1975; Martonosi et al., 1972). Phosphoenzyme formation following modification by DEPC has not been uniformly reported by other investigators. However, it is also evident in Fig. 4 that the ability to form a DEPC-phosphoenzyme depends on the careful quenching of the reaction at a point where histidyl modification is nearing completion and the inhibition of phosphoenzyme formation is limited. Certainly if longer incubation times were used or if higher concentrations of reagent were used, a different result would be obtained. A second factor which may affect phosphorylation experiments, as well as ATP binding studies (Tenu et al., 1976b), is the high reactivity of DEPC toward ATP, so that DEPC-enzyme preparations must either be exhaustively washed or excess DEPC removed by reaction with another reagent, before experiments can be performed.

To ensure that histidyl modification was responsible for the loss in hydrolytic activity, the modified enzyme preparation was incubated in hydroxylamine. Hydroxylamine will exchange exclusively with the N-1 nitrogen on the histidine ring (Melchior and Fahney, 1970) and this technique was used universally to align loss of functional properties with histidine modification in the recent DEPC studies cited above. As is shown in Fig. 4, the major fraction of the ATPase activity returned following incubation with hydroxylamine. The fraction of activity that could not be reclaimed corresponds closely to the fraction of the enzyme that loses the ability to form E-P.

**Effect of DEPC Modification on Ca²⁺ Binding**—It is well established that approximately 8 nmol of Ca²⁺ bind with high affinity (Kₐₙ = 2 μM) to 1 mg of the ATPase and in doing so activate the enzyme (Inesi et al., 1980). Ca²⁺ binding curves are shown in Fig. 5a. Size exclusion chromatography was used to equilibrate given solutions of ⁴⁰Ca²⁺ with vesicular SR and samples were run back to back on DEPC-SR and normal SR which had been treated in exactly the same manner. While the normal binding curve exhibits the high cooperativity (Hill coefficient = 1.8) which is characteristic of the ATPase, it is evident in the figure that the DEPC SR binding curve does not exhibit the steep slope associated with cooperative bind-
DEPC Modification of the SR ATPase

Fig. 5. Calcium binding to high affinity sites on DEPC-ATPase as a function of free Ca²⁺. a, Ca²⁺ binding to DEPC-ATPase determined by size exclusion chromatography (○, ●) and EPR measurements (△) is compared to that of the normal ATPase (□, by chromatography only). Solid line gives the best fit to the DEPC-ATPase data for two independent sites with \( K_n = 14 \, \mu M \), \( N_1 = 5.3 \, \text{nmol/mg} \), \( K_d = 0.5 \, \mu M \), and \( N_2 = 3.6 \, \text{nmol/mg} \). Dashed line gives the best fit to the normal ATPase data for a cooperative curve with Hill coefficient = 1.8, \( K_n = 10 \, \mu M \), \( N_0 = 8.4 \, \text{nmol/mg} \), and \( N_{tot} = 8.8 \, \text{nmol/mg} \). Dashed line indicates points where small subtractions were made for nonspecific binding. b, The DEPC-ATPase data is given in the form of a Scatchard plot. The dashed line compares the best fit for a one site model with \( K_n = 1.4 \, \mu M \) and \( N_{tot} = 8.1 \, \text{nmol/mg} \). The EPR parameter \( SC/B = [C/B - C/B (at Ca^n = 0)]/[C/B (at Ca^n = 10 \, \mu M)] - C/B (at Ca^n = 0) \). The line heights C and B are defined in the text and in Coan (1983).

The steep nature of the cooperative curve allows the high affinity sites to be clearly separated from a large number of nonspecific sites with low binding affinities \( (K_{app} \approx 1 \, \text{nmol}) \) which originate from various sources in the SR vesicles (Inesi et al., 1980). This is not the case with the DEPC-SR, as the high end of the noncooperative curve falls under the tail of the sites which are beginning to saturate in the millimolar range. We have resolved this portion of the binding curve (last three points in Fig. 5a) by subtracting the contribution from the nonspecific sites, using the binding data from the normal SR curve to estimate this contribution. We have also followed Ca²⁺ binding effects on the EPR spectrum of spin-labeled SR. It has been demonstrated for normal SR vesicles that a conformational change which accompanies Ca²⁺ binding to the Mg-ATP-ISL-ATPase complex alters the motional parameters of an ISL which is covalently attached to the enzyme (Coan and Inesi, 1977). As only binding which affects the enzyme conformation alters the spectrum, this technique can be used to discern the shape of the binding curve through the millimolar range of \( [Ca^{2+}] \) (Coan et al., 1979). As is shown in the figure, the change in ISL parameters also follows the Ca²⁺ binding curve of the DEPC-ATPase, with the added advantage that the full curve may be resolved. The actual change observed in the EPR spectrum is produced by the splitting of the major spectral band into two components, one of which has greatly restricted motion. Nucleotide must be present to maximize this effect and we have used AMP-PCP to eliminate turnover during measurement. The parameter, \( C/B \), is the ratio of two spectral lines which are particularly sensitive to the splitting (Coan and Keating, 1982; Coan, 1983). Modification of the ISL-ATPase by DEPC did not alter the pattern of spectral change.

Fitting procedures were then used to obtain estimations of binding constants and binding stoichiometries. The best fit to the DEPC-ATPase binding curve (solid line, Fig. 3a) was given by two independent sites, with \( N_1 = 5.3 \, \text{nmol of Ca}^{2+}/ \text{mg protein} \), \( K_n = 14 \, \mu M \), \( N_2 = 3.6 \, \text{nmol of Ca}^{2+}/\text{mg protein} \), and \( K_d = 0.5 \, \mu M \). For comparative purposes, the data was also fit for one overall binding constant. The total binding stoichiometry \( (8.9 \, \text{nmol of Ca}^{2+}/\text{mg protein}) \) is that of two Ca²⁺ per ATPase chain, and it is well established that two Ca²⁺ are transported for every ATP hydrolyzed, so that this model would represent two independent Ca²⁺ sites with the same apparent binding affinity. The fit for this model was clearly not as good as that of the two-site model. The discrepancy is more apparent when the data is plotted in the form of a Scatchard plot (Fig. 5b, solid line versus broken line). Furthermore, the curvature observed in the experimental data over the full range of the Scatchard plot is good evidence that more than one binding constant contributes to the function.

The best fit to the binding curve for the normal ATPase, which was measured concomitantly with the DEPC-ATPase curve, was obtained with a two site cooperative binding model with \( K_{dl} = 10 \, \mu M \) and \( K_d = 0.4 \, \mu M \), and a maximal binding of \( 9.8 \, \text{nmol/mg} \) SR protein. It is interesting that the binding constants which give the best fit to the independent site model for the DEPC-ATPase are very close to those obtained for the cooperative fit for the normal ATPase and it appears that while DEPC modification has eliminated cooperativity in the binding, it has not significantly altered the site specific binding constants.

Relation of Ca²⁺ Binding to E-P Formation on DEPC-ATPase—Since the DEPC-modified enzyme can still be phosphorylated by ATP, and Ca²⁺ binding is normally a stringent requirement for phosphorylation, this system should provide an opportunity to independently observe the effect of Ca²⁺ binding to each of the two sites on E-P formation. By quenching the DEPC reaction at 20 min we should obtain preparations that are largely modified but still retain the ability to form E-P. A titration of E-P formation as a function of Ca²⁺ concentration is given in Fig. 6a for these preparations. Theoretical curves generated by the separate binding constants for Ca²⁺, which were obtained from the fitting procedures for the two independent site model and for the cooperative binding model, are also given. As is evident in the figure, E-P formation follows quite closely to the curve predicted by the binding constant of the independent site with the highest affinity for Ca²⁺. It should also be noted that because Ca²⁺ is required for activation, these measurements give an independent means of observing Ca²⁺ binding, and the good agreement clearly establishes the presence of one independent high affinity site on the modified ATPase. However, the E-P which is formed is apparently very slow to hydrolyze, and it could be possible to obtain maximal levels of E-P through a shift in equilibrium. For instance, it is possible that Ca²⁺ binding to the low affinity site, or both sites, is required for E-P formation. Only a very small fraction of the low affinity site would be saturated at a given time, but the E-P, once formed, being slow to hydrolyze, could build up to maximal levels under steady state. For this reason we have also measured the rate of E-P formation at given Ca²⁺ concentrations. A less than saturating level of ATP \( (2 \, \mu M) \) was used to slow the reaction to where it could be measured with our filtration techniques, and under these conditions the apparent rate of E-P formation would vary between one and 2 orders of magnitude over the

\[ \text{Ca}^{2+} \text{ Bound/ Ca}^{2+} \]
DEPC Modification of the SR ATPase

FIG. 6. E-P formation as a function of Ca$^{2+}$ concentration for DEPC-ATPase. a, percent of E-P formed from $[^{32}P]ATP$ is compared to theoretical binding curves generated for independent sites with $K_d = 14$ $\mu M$ and $K_d = 0.4$ $\mu M$, respectively (solid lines), and to the cooperative curve given in Fig. 2a (dashed line). b, the rate of formation of E-P is given for the Ca$^{2+}$ concentrations indicated in the figure. For DEPC-ATPase, 100% E-P = 2.5-3.0 nmol/mg. To obtain rate measurements the $[^{32}P]ATP$ concentration was reduced to 2 $\mu M$. Otherwise conditions are as given under "Experimental Procedures."

The most interesting information concerning the ATPase may come from observing which steps in the mechanism are altered, which remain intact, and how they are related, rather than from the direct observation of the inhibition. In fact, we cannot say that histidine is directly involved in any given step in the mechanism on the basis of modification data alone. The types of changes that we see here could easily be due to localized denaturation in sensitive areas of the structure. For instance, we see the cooperative aspect of the Ca$^{2+}$ binding mechanism to be lost concomitantly with inhibition, while the direct relationship between Ca$^{2+}$ binding and enzyme phosphorylation remains. The very nature of cooperativity requires an element of change in the enzyme conformation, and if this change has a functional role in the mechanism other than to control the binding of a second Ca$^{2+}$, it appears that it is not required for the activation process. It is possible that such a change may contribute to the proper utilization of the energy held within the acyl phosphate bond for transport. This would certainly be in line with the observed inhibition of E-P hydrolysis which could easily be included by a degree of denaturation involving one or several His residues in pertinent areas of the structure.

One very specific result of DEPC modification, however, is that it allows us to look at the binding of each Ca$^{2+}$ separately, and to establish appropriate binding constants. Such constants are difficult to observe when cooperatively is present, and these studies give the first direct experimental observation of the individual sites on the ATPase under conditions where high affinity is maintained. Furthermore, these are the first studies to show a direct relationship between Ca$^{2+}$ binding at a given site and enzyme phosphorylation. Accordingly, they should provide insights into these respective mechanisms which cannot otherwise be obtained.

Two important questions in any cooperative mechanism concern the initial relationship between the binding sites and the type of change which occurs in the enzyme to induce the cooperativity. In fact, cooperativity is usually defined by the degree of change in apparent binding affinity which follows binding to an initial site. However, the DEPC modification studies show that when the cooperativity has been eliminated the site-specific affinities remain, apparently as an intrinsic property of the enzyme. Both observations can be reconciled by at least two different types of mechanisms. In general, an apparent binding constant can be described as the product of the site-specific binding constant and the statistical availability of the site (Coan et al., 1975), so that a mechanism in which a conformational change associated with cooperativity exposes or otherwise makes available, an occluded site with an established affinity, could certainly explain the data if the result of the His modification was simply to open up access to a second binding domain. Such a mechanism would certainly be in line with the putative location of the Ca$^{2+}$ sites within an intermembrane channel (Clarke et al., 1989). Alternately, if a histidine were directly involved in the binding mechanism, for instance if cleavage of a His hydrogen bond was required to induce high affinity to the second site, the modified enzyme would remain in the cleaved high affinity state due to the formation of the N-carbethoxy bond. This mechanism would be in line with observed pH effects (Inesi and Hill, 1983) since at high pH (where His would not be protonated) the highest degree of cooperativity is found. It is most important, however, that a Monod-Wyman type of mechanism (in which the Ca$^{2+}$ sites are of equivalent affinity, an apparent cooperativity being induced by a pre-binding shift in equilibria between two enzymatic states) cannot be supported by our data. Similarly, a mechanism in which apparent cooperativity is induced by a conformational change...
that follows binding to two equivalent sites would not be supported by our data, although, as Tanford and coworkers (Tanford et al., 1985, 1987) have pointed out, such a mechanism can be used to explain many of the equilibrium binding studies and kinetic steady state measurements on the ATPase.

Both mechanisms described above as being consistent with the modification data are of a sequential nature, and in fact, the large difference in given binding affinities that we observe for the two Ca^2+ sites cannot be explained adequately by another type of mechanism. Evidence for sequential Ca^2+ binding has been previously observed at low temperatures (Ikemoto et al., 1981; Dupont, 1982), however, it is still most reassuring that rapid kinetic measurements have recently produced direct evidence of sequential binding under ambient conditions (Petithory and Jencks, 1988a). In addition to these studies, which focused on the initial binding process, it has recently been demonstrated that Ca^2+ is also released by the enzyme in sequential fashion both to the external medium from the initial binding state (Petithory and Jencks, 1986b; Inesi, 1987; Nakamura, 1986) and to the vesicle interior following phosphorylation (Khananshvili and Jencks, 1988; Inesi, 1987; Nakamura, 1987). All three groups of investigators cited evidence which would indicate that one Ca^2+ site was more occluded (e.g., less accessible for isoform exchange or EGTA chelation) than the other, which has also been observed in terms of water accessibility to the bound Ca^2+ (Klemens et al., 1986). However, Inesi (1987) was further able to demonstrate that it is the less accessible Ca^2+ which is the first to be released to vesicle interior following enzyme phosphorylation, while both Jencks and coworkers (1988b) and Klemens and coworkers (1986) were able to demonstrate that the less accessible Ca^2+ has the higher affinity of the two. These observations are clearly in accord with the data presented here, and taken together they suggest that a mechanism in which site accessibility controls cooperativity is a very reasonable model.

The second, and perhaps most important, result of the DEPC modification studies is the direct alignment of enzyme phosphorylation with Ca^2+ binding to the site with highest affinity. As discussed above, this must necessarily be the second site to bind Ca^2+ in a sequential cooperative mechanism and, according to the work of Jencks and Inesi, would be the most occluded site. Several observations then follow. First, the placement of E-P formation at this point in the cycle clearly indicates a requirement for two Ca^2+; binding of the first Ca^2+ controls the binding of the second (normally through the change in conformation associated with cooperativity) and the second Ca^2+ triggers phosphoenzyme formation. Second, some type of intramolecular rearrangement is required after the second Ca^2+ binds to make the connection between the binding and the phosphorylation process, and it is precisely at this step in the cycle that Jencks and coworkers observe a slow, and under certain conditions rate limiting, step in the mechanism (Petithory and Jencks, 1986, 1988a).

While it is clear in the studies of Jencks and coworkers that this putative conformational change is a first order phenomenon which follows Ca^2+ binding, but precedes the actual transfer of the γ-phosphate, it has not been clear whether Ca^2+ binding to both sites or just the second site is required. Clearly our data indicate that binding to a second site is sufficient in itself to fully induce phosphorylation. It is also clear that this change is distinct from that associated with binding cooperativity, as the modified enzyme will form maximal levels of E-P.

On the basis of their kinetic studies, Tanford et al. (1987), Inesi (1987), and Khananshvili and Jencks (1988) have proposed sequential binding models with many similar features, and while the model of Tanford and coworkers did not require that Ca^2+ binding be cooperative, it did require that enzyme phosphorylation follow binding of the second Ca^2+ in sequential order, which would strongly be supported by our studies as well as those of Jencks and coworkers. Both Inesi (1987) and Tanford and coworkers (1987) suggested that Ca^2+ binding to an initial site may create a second site to accommodate the sequential aspects of the mechanism. Alternatively, the recent model suggested by Clarke et al. (1989), taken together with the data presented here, gives at least equal credence to site exposure as a means of inducing the sequential aspects of the mechanism. In either case, it now appears almost certain that Ca^2+ binding to the high affinity sites on the ATPase is truly cooperative and sequential in nature, that enzyme phosphorylation is triggered by Ca^2+ binding to the second site which also appears to be highly occluded, and, on the basis of the work of Inesi (1987), appears to be the first to release Ca^2+ to the vesicle lumen following enzyme phosphorylation.

Acknowledgments—We thank Gail Cassafer for her expert technical assistance and Drs. Richard Coll and Alexander Murphy for helpful discussions throughout the preparation of this manuscript.

REFERENCES
Effect of diethyl pyrocarbonate modification on the calcium binding mechanism of the sarcoplasmic reticulum ATPase.
C Coan and R DiCarlo


Access the most updated version of this article at [http://www.jbc.org/content/265/10/5376](http://www.jbc.org/content/265/10/5376)

**Alerts:**
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/265/10/5376.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/265/10/5376.full.html#ref-list-1](http://www.jbc.org/content/265/10/5376.full.html#ref-list-1)