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(Iodoacetamido)fluorescein Labels a Pair of Proximal Cysteines on the Ca^{2+}-ATPase of Sarcoplasmic Reticulum†

James E. Bishop,* Thomas C. Squier, Diana J. Bigelow, and Giuseppe Inesi

Department of Biological Chemistry, University of Maryland School of Medicine, 660 West Redwood Street, Baltimore, Maryland 21201

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ABSTRACT: Previous energy transfer studies [Squier, T. C., Bigelow, D. J., de Ancos, J. G., & Inesi, G. (1987) *J. Biol. Chem. 262*, 4748–4754] have utilized fluorescent iodoacetamide derivatives covalently bound to the Ca^{2+}-ATPase of sarcoplasmic reticulum (SR), using labeling conditions that completely modify the most reactive of the protein's surface sulfhydryls to a final level of 9 nmol/mg of SR protein. Unambiguous interpretation of these results requires localization of these labeling sites with respect to the primary structure of the Ca^{2+}-ATPase. In the present study, we have used the probe 6-(iodoacetamido)fluorescein (IAF) as a marker for these sites. The IAF-labeled Ca^{2+}-ATPase was completely proteolyzed with trypsin, followed by centrifugation to remove (unlabeled) membrane-associated portions. The soluble IAF-labeled tryptic peptides were purified by size-exclusion and reverse-phase high-performance liquid chromatography. Two IAF-peptides resulted. The major (4.1 nmol of IAF/mg of starting protein) and minor (1.9 nmol/mg) IAF-peptides were sequenced and were identified, respectively, as Ala_{673}-IAF-Cys_{674}-Cys_{675}-Phe_{676}-Ala_{677}-Arg_{678} and as Glu_{668}-Ala_{669}-IAF-Cys_{670}-Arg_{671}. A model is proposed to explain the selectivity of IAF for Cys_{670} and Cys_{674} of the ~14 surface sulfhydryls of the Ca^{2+}-ATPase. The labeling region, Arg_{667} through Arg_{678}, has been predicted to be α-helical; Cys_{670} and Cys_{674} would be adjacent in the helix and imbedded in an Arg cluster. The Arg residues would both attract the anionic IAF and enhance sulfhydryl reactivities by lowering their pK values.

The Ca^{2+}-ATPase of sarcoplasmic reticulum (SR)[1] has been extensively studied to determine the kinetic mechanism of ion transport and, more recently, the causative structural features thereof [see Inesi (1985) for a recent review]. One widely used avenue has been through the use of sulfhydryl-directed spin-label and fluorescence reagents, primarily iodoacetate and maleimide derivatives, which modify a limited number of the surface cysteinyl residues of the protein under certain conditions [see Ikemoto (1982) for a review].

Of the 24 cysteinyl residues of the Ca^{2+}-ATPase (Brandl et al., 1986), roughly 14 are on the surface of the protein and accessible to modification reagents (Murphy, 1976, 1978; Thorley-Lawson & Green, 1977; Ikemoto et al., 1978; Reithmeier & MacLennan, 1981). These authors have found that the 14 cysteines can be grouped into about 3 reactivity classes spanning a range of 1–2 orders of magnitude in their chemical reactivities to different sulfhydryl-directed reagents. Microenvironmental influences presumably account for the differing reactivities. Selective modification of only one to three cysteinyl residues per Ca^{2+}-ATPase has been achieved, in all cases, by using a lower pH (6.0–7.0), where the difference between reactivity classes is more marked and, in some cases, by using limiting amounts of modifying reagent. Some sulfhydryl-directed probes used to study the Ca^{2+}-ATPase have included the following: conformationally sensitive iodoacetamide and maleimide spin-labeles (Landgraf & Inesi, 1969; Coan & Inesi, 1977; Coan et al., 1979; Coan & Keating, 1982; Yasuoka-Yabe et al., 1983); conformationally sensitive fluorescent probes, such as (anilinonaphthyl)maleimide, [(benzimidazolyl)phenyl]maleimide, [[(dimethylamino)-methyl]coumarinyl]maleimide, and pyrenylmaleimide (Miki et al., 1981; Yasuoka-Yabe & Kawakita, 1983; Yasuoka-Yabe et al., 1983; Kurtenbach & Verjovski-Almeida, 1985); and fluorescent probes used for distance measurement, such as

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[1] Abbreviations: SR, sarcoplasmic reticulum; IAF, 6-(iodoacetamido)fluorescein; IAF–SR, covalent adduct from reaction of IAF with SR; IAEDANS, 5-[[2-(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; AUFS, absorbance units full scale; FITC, fluorescein isothiocyanate.

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IAF, IAEDANS, and pyrenylmaleimide (Vanderkooi et al., 1977; Ludi & Hasselbach, 1983; Papp et al., 1986; Fagan & Dewey, 1986; Squier et al., 1987).

Building upon the foundation of predicted two-dimensional secondary structural models [e.g., see Brandl et al. (1986)], we have recently initiated studies to construct tertiary structural models of the Ca^{2+}-ATPase with measurements of resonance energy transfer from the donor, IAEDANS, to other discretely modified residues on the protein (Squier et al., 1987). To this end, it is essential to know the precise residue or residues modified with this fluorescent iodoacetamide probe with respect to the primary structure. We have therefore modified the Ca^{2+}-ATPase with a more highly absorbant marker of these sites, IAF, under the restrictive conditions described above. We have shown that it labels the same reactive residues as does IEADANS and have localized the labeling to a pair of proximal cysteinyl residues. Further, a secondary structural model of this region of the protein offers an explanation for the enhanced reactivity of these two cysteines toward polar probes such as IAF. This work was presented in preliminary form (Bishop et al., 1987b).

Materials and Methods

Materials. Bovine pancreatic trypsin [treated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone] and iodoacetic acid were from Sigma (St. Louis, MO). Methanol (HPLC grade) was from J. T. Baker Chemical Co. (Phillipsburg, NJ). IAF was from Molecular Probes (Junction City, OR). The purity of the IAF was determined to be ~96% by reverse-phase HPLC.

Standard Methods. SR vesicles were isolated from rabbit hind leg white muscle (Eletr & Inesi, 1972). Protein concentrations were determined by the method of Lowry et al. (1951) or by absorbance, using $\epsilon_{280} = 10.5\ cm^{-1}$ for 10 mg of SR protein/mL in 1% sodium dodecyl sulfate (Thorley-Lawson & Green, 1977). The Ca^{2+}-ATPase content of SR vesicles was determined to be ~44% of the SR protein from determinations of optimal enzyme phosphorylation (Bishop et al., 1987a). Limited tryptic cleavage, denaturing polyacrylamide gel electrophoresis (Weber & Osborn, 1969), visualization of IAF on these gels, and fluorescence measurements were done as before (Squier et al., 1987).

Labeling and Quantification of IAF. SR vesicles were labeled with IAF as was described for IAEDANS labeling (Squier et al., 1987). In brief, this entailed reaction at pH 6.8 at 25 °C for 30 min with a 25-fold molar excess of IAF over Ca^{2+}-ATPase; unreacted probe was removed by Sephadex G-50 column chromatography and centrifugation of the SR vesicles at 40000*g* for 45 min. The IAF-labeled SR vesicles were resuspended in 10% sucrose and stored frozen at −20 °C.

The amount of IAF covalently bound to SR protein was quantified by the absorbance at pH 8.5 (NH_4HCO_3 buffer) in 1% sodium dodecyl sulfate using $\epsilon_{495} = 75\ mM^{-1}\ cm^{-1}$ (Eshaghpour et al., 1980). The absorbance value of authentic IAF was not altered by the presence or absence of 1% sodium dodecyl sulfate or by the use of 0.1 N NaOH rather than NH_4HCO_3 buffer. There were no obvious changes in the spectral properties of IAF upon covalent attachment to SR protein when measurements were made with the denatured protein.

Soluble Tryptic Peptides from IAF-SR. The reaction mixture, in a volume of 400 μL, contained 50 mM NH_4HCO_3 (pH 8.5), 100 μM $CaCl_2$, 1 mM dithiothreitol, 2.0 mg of IAF-SR, and 0.02 mg of trypsin. Incubation was for ~4 h at 37 °C. Five millimolar iodoacetic acid was then added, and incubation was continued for 1 h. The mixture was diluted with ice-cold H_2O to a volume of 2.5 mL and centrifuged at 40000*g* for 1 h in polysulfone tubes. The supernatant was analyzed for peptide and covalent IAF content by absorbance measurements, respectively, at 214 and 495 nm. It was then dried on a Savant Speed-Vac concentrator for later purification by HPLC.

The above conditions were optimized for quantitative formation of small peptides. The use of dithiothreitol and a temperature of 37 °C, rather than 25 °C, for the trypsinolysis were instrumental in increasing the release of IAF label from the SR membrane from 80–90% to 95–100% and also in producing quantitative cleavage at a partially occluded tryptic site near the major IAF labeling site. The latter observation was made in size-exclusion HPLC experiments (cf. Figure 2) in which a somewhat larger IAF-peptide peaking at 10.7 min was converted to the 11.2-min peak observed in Figure 2. Digestion times between 1 and 16 h gave essentially the same pattern of peptides seen on HPLC separations (e.g., Figures 2 and 3). The $CaCl_2$ was included to protect trypsin from autodigestion (Buck et al., 1962). Carboxymethylation of free sulfhydryls with iodoacetate resulted in a better recovery of peptides from HPLC columns and was also necessary to protect cysteines from degradation during sequencing.

HPLC. Peptide separations utilized a Waters HPLC system, equipped with a 250-μL injection loop, a Waters Model 441 absorbance detector for far-UV wavelengths, and an Isco UA-6 absorbance detector for visible wavelengths. Columns used were as follows; TSK-G2000SW, 7.5 mm × 30 cm, manufactured by ToyoSoda Co.; C_3 Ultrapore, 4.6 mm × 7.5 cm, from Beckman Altex Co.; and Vydac C_{18}, type TP silica, 4.6 mm × 15 cm, from Separations Group (Hesperia, CA). The silica particles of both reverse-phase columns had average pore sizes of 300 Å.

Dried peptide samples for injection (amounts are given in the figure legends) were dissolved in the buffer in which the column was equilibrated, and 100 μL was injected. Where gradient elution was utilized, a linear gradient over 30 min was initiated at the sample injection point with flow rates of 0.5–1.0 mL/min as given in the figure legends. IAF-peptide absorbance was monitored at 435 nm, where the extinction coefficient in pH 5.5 solutions was determined to be $15\ mM^{-1}\ cm^{-1}$. Eluant absorbances were recorded on a strip recorder running at 0.5 cm/min; the two pens were offset, with the 435-nm pen leading the 214-nm pen by 0.5 cm. The elution volume noted on the abscissa of figures was aligned with the 435-nm pen. Fractions of 0.4 mL were collected; their absorbances were read with an Aminco-SLM DW-2 spectrophotometer to monitor recovery from columns.

Peptide Sequencing. Sequencing was provided by Dr. William Brown at the Centralized Facility for Protein Analysis at Carnegie Mellon University. The procedure utilized automated phenyl isothiocyanate chemistry on an Applied Biosystems gas-phase sequencer, Model 470A (Hunkapiller et al., 1983; Hunkapiller & Hood, 1983).

Results

Specific Labeling of Ca^{2+}-ATPase in SR Membrane with IAF. IAF was chosen as a marker for the reactive sulfhydryls on the Ca^{2+}-ATPase, from which distance measurements were previously made with the probe IAEDANS (Squier et al., 1987), due to its high extinction coefficient, allowing sensitive detection during HPLC. Data discussed below show that IAF and IAEDANS modify the same sites. SR vesicles were reacted with the sulfhydryl-directed probe IAF under the moderate pH conditions generally used to modify only the most reactive of the ~14 surface sulfhydryls of the Ca^{2+}-ATPase

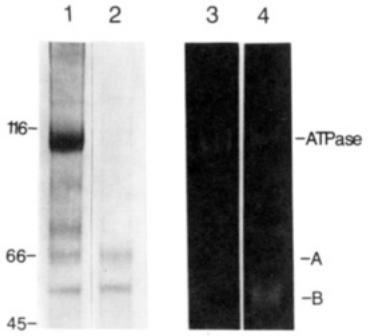

FIGURE 1: Gel electrophoresis of IAF-SR and its major tryptic fragments. IAF-SR (lanes 1 and 3) and IAF-SR which had been treated briefly with trypsin to produce the two major fragments, A and B (lanes 2 and 4), were separated on a 7.5% denaturing gel (Weber–Osborn). Visualization was with Coomassie Blue staining for protein (lanes 1 and 2) or by fluorescent light for IAF (lanes 3 and 4).

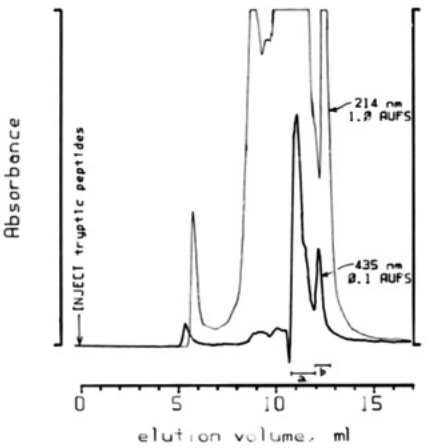

FIGURE 2: Separation of tryptic IAF-peptides by size-exclusion HPLC. Soluble tryptic peptides derived from 0.9 mg of IAF-SR in 100 μL of mobile phase were injected onto a TSK-G2000SW column (7.5 mm × 30 cm); elution was at 0.5 mL/min with a mobile phase consisting of 10 mM acetate (ammonium salt, pH 5.5) in 20% methanol. Peptide and IAF were monitored, respectively, by the absorbance at 214 and 435 nm. Fractions were pooled as indicated by the horizontal bars for further purification. Recovery of IAF material, determined by absorbance of the collected fractions, was 90–95% of that injected.

as has been documented for IAEDANS (Squier et al., 1987). To ensure complete labeling of these reactive residues, reaction media contained an excess of IAF, i.e., routinely, 100 nmol/mg of SR protein (25 mol/mol of Ca^{2+}-ATPase). Covalent modification was with a single rapid phase of reaction which saturated at a level of 9.0 ± 1.0 nmol/mg of SR protein. This level represented a plateau in time course experiments, even when the probe concentration was increased to as much as 1000 nmol of IAF/mg of SR protein. The least heterogeneity of labeling (as determined by HPLC separations shown later; e.g., Figure 3) was obtained when the reaction was conducted at 25 °C, rather than at 0 °C, and when new batches of IAF, obtained from Molecular Probes, were used.

The labeling level of 9 nmol of IAF/mg of SR protein would represent about 2 mol of IAF/mol of Ca^{2+}-ATPase, where the Ca^{2+}-ATPase concentration in our preparations of SR vesicles was determied by phosphorylation of the protein to levels of 4 nmol/mg of SR protein [see Barrabin et al. (1984) and Bishop et al. (1987a)]. Experiments were conducted as a preliminary characterization of the labeling and as a testing of the hypothesis that two sites were labeled. It was found that IAF and the probe IAEDANS were mutually competitive for labeling of SR, with either probe alone or both together reaching a maximum labeling level of about 9 nmol/mg; this showed that the two probes modified the same sites. In an experiment in which the SR was labeled with IAEDANS to different levels between 1 and 9 nmol/mg of protein, the IAEDANS fluorescence was found not to be a linear function of the labeling level but rather a sigmoidal function, thus suggesting two (or more) labeling sites with different reactivities and microenvironments. Finally, measurement of resonance energy transfer from IAEDANS to IAF, utilizing SR which had been simultaneously reacted with both probes, showed that the probes were attached within their static limit, i.e., within 25 Å of each other. Further, this static quenching was not altered by inclusion of the detergent lysolecithin, showing that the two labeling sites were on the same peptide chain. Collectively, these experiments could not rule out the presence of more than two labeling sites whose collective sum was about 2 mol of modified Cys/mol of Ca^{2+}-ATPase but do show clearly that there were at least two discrete sites within 25 Å of each other, thus defining a localized region on the Ca^{2+}-ATPase which is highly reactive toward these two iodoacetamide derivatives.

Limited Trypsinolysis of IAF-SR. Separation of the IAF-labeled SR by denaturing gel electrophoresis (Figure 1, lane 1) showed that the majority of IAF comigrated with the Ca^{2+}-ATPase (molecular weight 110000). A small amount of label comigrated with an M_r 80000 band (lanes 1 and 3); this species was formed in the presence of IAF and may be an intramolecular cross-link of the Ca^{2+}-ATPase.

Limited proteolysis [see Inesi (1985)] of the IAF-labeled SR with trypsin to produce the major fragments (Figure 1, lanes 2 and 4) showed all of the SR label to be localized to the B fragment at 55000 daltons, with none on the A fragment. These results constrained the IAF labeling sites to the B fragment of the Ca^{2+}-ATPase (Thorley-Lawson & Green, 1973), now known to include residues 506–1001 (Brandl et al., 1986).

Complete Trypsinolysis of IAF-SR. Determination of the precise residues labeled by IAF required cleavage of Ca^{2+}-ATPase into small peptides which could be purified and sequenced. Extensive treatment with trypsin should produce 103 peptides ranging in length from 1 to 123 residues based upon the known number of Lys and Arg residues (Brandl et al., 1986). Such treatment, followed by high-speed centrifugation to pellet the SR membranous components, resulted in the solubilization of 75% of the SR protein as peptides (see Materials and Methods). Electron microscopic observations (Scales & Inesi, 1976) have shown that trypsin removes the globular cytoplasmic portion of the protein away from the intramembranous portion; it was therefore concluded that supernatant protein was derived from the former domain and that in the pellet, from the latter. This trypsinolysis also resulted in the solubilization of 98% of the IAF label. In conjunction with the data shown in Figure 1, it was concluded that the IAF labeling sites were on the cytoplasmic portion of the B tryptic fragment.

This protocol eliminated the intramembranous sequences of Ca^{2+}-ATPase which are notoriously difficult to solubilize [see Green and Toms (1985)] and likewise eliminated the need to delipidate the sample before injection onto HPLC columns. The resultant supernatant tryptic peptides were purified by HPLC as detailed below.

Purification of IAF-Peptides by Size-Exclusion HPLC. Initial purification was by size-exclusion HPLC on a TSK G2000SW column (Figure 2). Elution of peptides (and other reagents) was followed by far-UV absorbance at 214 nm. The

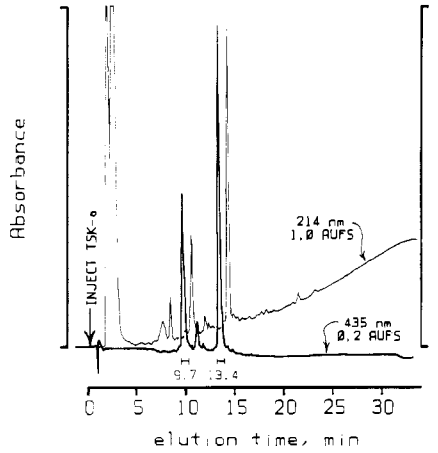

FIGURE 3: Separation of size-exclusion column fractions by reverse-phase HPLC on a C_3 column at pH 5.5. Fraction TSK-a, pooled from three identical runs as shown in Figure 2, was injected in a volume of 100 μL onto a C_3 column (4.6 mm × 7.5 cm). Elution was at 1.0 mL/min with 10 mM acetate (ammonium salt, pH 5.5) with a linear 30-min gradient from 0% to 95% methanol initiated at the injection point; the course of the gradient is marked by the increasing 214-nm base line due to the absorbance of methanol. Peptide and IAF were monitored, respectively, by the absorbance at 214 and 435 nm. Fractions of 0.4 mL were collected, and those indicated by the horizontal bars at 9.7 and 13.4 min were retained for further purification. Recovery of IAF absorbance was 80–90% of that injected.

column void was at 5.4 mL; trypsin eluted at ~6.1 mL (determined in a separate experiment); iodoacetate was unexplainably retained to a certain extent by the column, eluting as a broad peak between 9.5 and 11.0 mL, causing the artifactual negative absorbance change in the 435-nm trace at 10.6 mL; and excess dithiothreitol eluted at the included volume, 12.3 mL (Figure 2).

Elution of IAF-labeled peptides was monitored at 435 nm (Figure 2). The peak at the void volume, 5.4 mL, was largely artifactual and contained ~1% of the recovered IAF absorbance; its size increased with column usage and was minimized by cleaning the column. The low broad peaks between 8.5 and 10.7 mL constituted 7–15% of the recovered IAF; this fraction was refractory to further trypsinolysis, gave no discrete peaks when rechromatographed on a C_3 reverse-phase HPLC column (cf. Figure 3 below), and was not analyzed further. The major fraction containing IAF label (designated TSK-a) eluted relatively late from the column at 11.2 mL; including the somewhat smaller molecular weight shoulder at 11.5 mL, this fraction contained 58–72% of the recovered IAF absorbance. The fraction, designated TSK-b, which coeluted with dithiothreitol at the included volume of 12.3 mL, constituted 16–27% of the recovered IAF absorbance. The TSK-b fraction was purified by the same reverse-phase HPLC method as was used for TSK-a (cf. Figure 3 below); elution was at 11.2 min, being slightly more polar than authentic IAF at 11.8 min (data not shown). Sequencing of this fraction through eight cycles of degradation gave no identifiable amino acid derivatives. Its A_{214}–A_{495} ratio (0.50) was identical with that of unreacted IAF. This fraction was suggested to be the hydrolysis product of IAF.

Separation of tryptic peptides on the TSK column (Figure 2) served both to give estimates of relative molecular size and to separate out high molecular weight peptides which otherwise tended to irreversibly bind to, and clog, subsequent reverse-phase columns. The major IAF fraction thus derived, TSK-a, was taken for further purification, shown below.

Purification of TSK-a by Reverse-Phase HPLC on C_3 at pH 5.5. Higher resolution separation was achieved on a reverse-phase HPLC column. A pH value of 5.5 was chosen for the eluting buffer in that peptides would be fully ionized and therefore most soluble. The fraction, TSK-a, was loaded in aqueous buffer (10 mM ammonium acetate, pH 5.5) and eluted with a gradient of methanol from 0% to 95% over 30 min (Figure 3). It may be noted that a similar elution pattern was obtained in a comparative experiment in which elution was with a gradient from 0% to 50% over 30 min of the commonly used solvent acetonitrile.

The large far-UV-absorbing peak eluting at ~1.0 mL as unretained material (Figure 3) was iodoacetate, which had overlapped TSK-a in the previous separation. This was responsible for the small artifactual peak in the 435-nm trace at the same position (Figure 3).

Two major IAF-labeled peptides from the TSK-a fraction were separated on the C_3 column, at 9.7 and 13.4 min (Figure 3). In a separate experiment, it was found that the former peptide corresponded to the smaller molecular weight shoulder at 11.5 mL in the TSK separation and the latter to the major TSK peak at 11.2 mL (compare to Figure 2 above). These two peptides, at 9.7 and 13.4 min, were taken for final purification, below.

The small 435-nm peak at 11.2 mL (Figure 3) was identified as contaminating TSK-b fraction. As discussed above, this was probably the hydrolysis product of IAF.

Final Purification of IAF-Peptides by Reverse-Phase HPLC on C_{18} at pH 5.5. Experiments showed that IAF-peptides separated in Figure 3 could be successfully purified either by use of the same column (C_3) with elution at a lower pH (pH 2.1, with 0.1% trifluoroacetic acid), which would protonate carboxyl groups, or by use of the same elution pH (5.5) with a more apolar column (C_{18}). It may be noted that both of these conditions would result in stronger apolar interactions between peptide and column matrix. Interestingly, it was found that altering both conditions simultaneously, i.e., C_{18} column eluted at pH 2.1, did not successfully purify the peptides (results not shown).

Separation of the peptides on C_{18} at pH 5.5 (10 mM ammoniuim acetate) is shown in Figure 4. Elution was with a gradient of methanol from 0% to 95%. The minor and major IAF-peptides, which had eluted respectively at 9.7 and 13.4 min from the C_3 column, eluted respectively from the C_{18} column at 11.9 min (Figure 4A) and 16.2 min (Figure 4B). The denoted fractions from Figure 4 were taken for sequencing.

Sequencing and Identification of Purified IAF-Peptides. The IAF-peptides purified in Figure 4 were sequenced; results are presented in Table I. Both the major (16.2 min) and minor (11.9 min) IAF-peptides yielded no discernible PTH

Table I: Sequences of Purified IAF-Peptides

peptide elution (min)[a]	sequence[b] and assignment[c]
16.2	Ala-X-CM-Cys-Phe-Ala-Arg
	Ala_{673}-IAF-Cys_{674}-Cys_{675}-Phe_{676}-Ala_{677}-Arg_{678}
11.9	Glu-Ala-X-Arg
	Glu_{668}-Ala_{669}-IAF-Cys_{670}-Arg_{671}

[a] IAF-peptides, eluting at 16.2 and 11.9 min from the C_{18} columns shown in panels A and B, respectively, of Figure 4 were collected for sequencing. [b] Peptides (about 1 nmol of each, based upon the IAF content) were sequenced as given under Materials and Methods. "CM-Cys" stands for carboxymethyl-Cys, arising from treatment of tryptic peptides with iodoacetate; "X" represents a sequencing cycle for which no PTH-amino acid was detected. [c] Assignment of peptides to the Ca^{2+}-ATPase primary structure was made by comparison to the sequence published by Brandl et al. (1986).

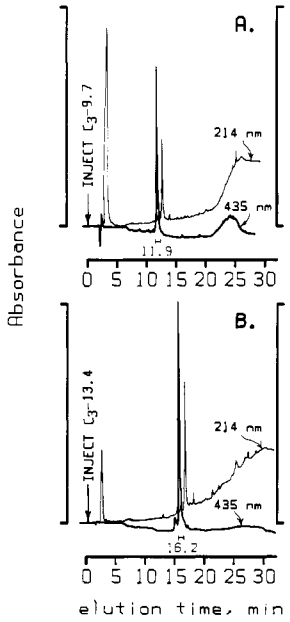

FIGURE 4: Final purification of IAF-peptides by reverse-phase HPLC on a C_{18} column at pH 5.5. Fractions from Figure 3, denoted as 9.7 and 13.4 min, were injected onto the C_{18} column (4.6 mm × 15 cm) in a volume of 100 μL (panels A and B, respectively). Elution was at 1.0 mL/min with 10 mM acetate (ammonium salt, pH 5.5) with initiation of a linear 30-min gradient from 0% to 95% methanol at the injection point. In (A) only, the gradient time was shortened from 30 to 10 min at the 22-min time point in order to hasten the finish of the run; the final [methanol] for both runs was 95%. Absorbance monitoring and fraction collection were as in Figure 3. Recovery of IAF absorbance was 50–90% of that injected; recovery values were progressively higher with column usage.

derivatives from the second and third degradation cycles, respectively. These residues were assumed to be IAF-Cys. The fluorescein moiety was apparently destroyed by the Edman degradation chemistry (probably by the trifluoroacetic acid), as no characteristic 495-nm absorbance was detected in alkalinized aliquots from these cycles.

The peptide sequence data (Table I) allowed unambiguous assignment of the two tryptic peptides to the primary sequence of the Ca^{2+}-ATPase (Brandl et al., 1986). The major peptide was identified as Ala_{673} through Arg_{678}, and the minor peptide was identified as the preceding tryptic peptide in the sequence, Glu_{668} through *Arg_{671}* (Table I). It may be noted that, consistent with the known specificity of trypsin, both peptides were preceded by Arg residues, Arg_{672} and Arg_{667}, respectively. It was concluded that the major and minor IAF labeling sites on Ca^{2+}-ATPase were, respectively, Cys_{674} and Cys_{670} (Table I).

IAF Labeling Stoichiometry. The generally good yields of IAF-peptides through fragmentation and purification procedures (above) allowed reasonable estimation of the original labeling stoichiometries of sites on the Ca^{2+}-ATPase. Most of the IAF label could be assigned to discrete sites. The major IAF site, Cys_{674}, labeled to the extent of 4.1 nmol/mg (Table II), about the same as phosphoenzyme intermediate levels generally observed with SR preparations from this laboratory; this suggested stoichiometric labeling of Cys_{674} [see Barrabin et al. (1984) and Bishop et al. (1987a)]. The secondary site, Cys_{670}, was only partially labeled by IAF (Table II), to the extent of about 0.5 mol/mol of Ca^{2+}-ATPase.

The moiety designated "free IAF" (Table II) could have been trapped inside the SR vesicles during the labeling reaction and retained despite extensive washing thereafter (see Materials and Methods). Alternatively, it is possible that, during subsequent processing and storage of the IAF-SR, some of the

Table II: Stoichiometry of IAF Labeling of the Ca^{2+}-ATPase

identity of IAF moiety	% of total label ± SD[a]	nmol/mg[b]
IAF-Cys_{674}	45.6 ± 4.2	4.1
IAF-Cys_{670}	20.8 ± 4.8	1.8
free IAF[c]	21.1 ± 3.7	1.9
other[d]	12.5 ± 3.2	1.2
	total 100	total 9.0

[a] Yields of the different moieties were calculated from measurements of IAF absorbance in fractions collected from the HPLC separation with respect to the known injection amount (Figures 2 and 3), assuming no selective loss of any one moiety. Tabulated data are averages from six separate experiments using three different IAF-SR preparations. [b] Calculated from the average of the percent distribution values and the IAF content of the starting IAF-SR, which was 9.0 ± 1.0 nmol of IAF/mg of SR protein (n = 5). [c] Material eluting at 12.3 mL from the size-exclusion HPLC column (Figure 2), postulated to be the hydrolysis product of IAF. [d] High molecular weight material eluting at 5.4 mL and between 8.5 and 10.7 mL from the size-exclusion HPLC column (Figure 2).

covalently attached IAF was slowly hydrolyzed. Such behavior was observed in a recent study using the related probe fluorescein isothiocyanate (Filoteo et al., 1987).

The last IAF moiety of Table II, "other", was high molecular weight material that was refractory to extensive trypsin digestion. It could not be purified or identified (see description associated with Figure 2).

Discussion

Determination of label stoichiometry in our previous resonance energy transfer study (Squier et al., 1987) presented evidence that more than one site may have been labeled by the sulfhydryl-directed fluorophore IAEDANS. It was therefore crucial for the unambiguous calculation of the site–site distances of that study to know if, indeed, there were multiple sites and their relative locations on the Ca^{2+}-ATPase. The present study has identified two labeling locations for the related probe, IAF, which was used as a high-sensitivity marker for the IAEDANS site(s) (see Results). In this study, we unambiguously identified two labeled sites on the Ca^{2+}-ATPase. The presence of both labels on the same polypeptide chain is supported by fluorescence measurements showing close interaction between the two sites using detergent-dispersed (monomeric) Ca^{2+}-ATPase (see Results) and by the 1.5 mol/mol labeling stoichiometry of the two sites per Ca^{2+}-ATPase chain (Table II). The primary and secondary labeling sites, respectively, are Cys_{674} and Cys_{670} (Tables I and II).

It would not have been possible to make accurate distance measurements from the donor fluorophore, IAEDANS, to other sites on the Ca^{2+}-ATPase (Squier et al., 1987) had the two IAEDANS labeling sites been far apart. This study shows that the two cysteine residues labeled by IAEDANS or IAF are indeed close together, being four residues apart in the primary structure of the protein and approximately 6 Å apart in the predicted secondary structure (see below and Figure 5). The spectral evidence suggesting a close proximity (see Results) is thus confirmed. In retrospect, we may now interpret the progressive increase of the IAEDANS quantum yield with increasing probe stoichiometry (Squier et al., 1987) as a direct interaction of the aromatic rings of adjacent fluorophores attached to the proximal cysteine residues. It is therefore reasonable to consider the donor fluorophores at the proximal cysteine residues as a point source with respect to the large distances measured to nucleotide site acceptors (56–68 Å). The shorter distance measured to acceptors at the calcium transport sites (16–18 Å) may be altered somewhat by the 6-Å separation of the two donor fluorophores at the proximal

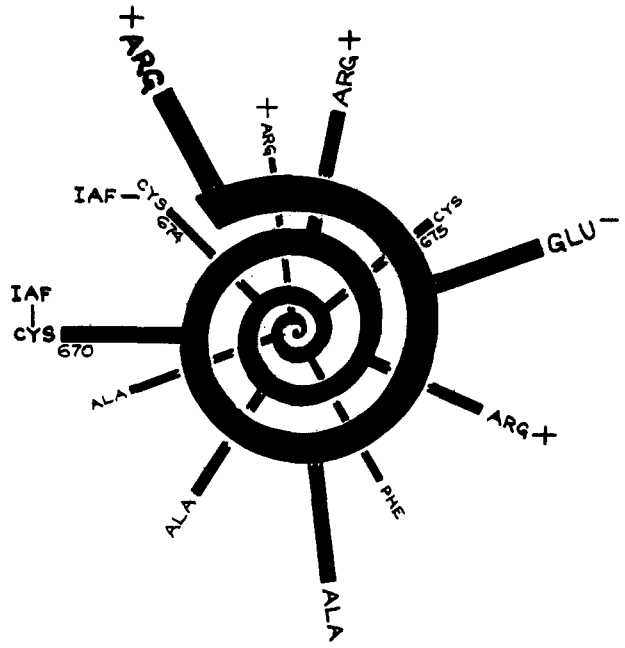

FIGURE 5: Secondary structural model of the peptide region containing IAF-labeled cysteines-670 and -674. The representation shows Arg_{667} through Arg_{678} in an α-helix spiralling inward and into the page.

cysteine residues, but this does not alter the basic aspects of our tertiary structural model of the Ca^{2+}-ATPase (see below).

While Cys_{674} was completely labeled (Table II), the half-labeling of Cys_{670} (Table II) might be accounted for by a model in which one subunit of a dimeric Ca^{2+}-ATPase would have a completely occluded Cys_{670} while that of the other subunit would be completely exposed for modification. A model of this sort has been proposed to explain the biphasic labeling of Ca^{2+}-ATPase by iodoacetamide spin-label in the presence of calcium and an ATP analogue (Coan & Keating, 1982). Alternatively, Cys_{670} may have been fully labeled initially but during subsequent processing selectively lost its label to form free IAF (see Table II and associated discussion).

The two labeled Cys residues, Cys_{674} and Cys_{670}, fall within the region of the Ca^{2+}-ATPase that has been predicted to be the nucleotide binding domain of the globular cytoplasmic head portion of the protein (Brandl et al., 1986). While there are some sequences within this nucleotide binding domain that are highly conserved within this family of ion-transporting ATPases, these proximal Cys residues are generally not conserved. In the slow-twitch Ca^{2+}-ATPase, which is most closely related to the fast-twitch Ca^{2+}-ATPase studied here, only Cys_{670} is conserved, while Cys_{674} is not (Brandl et al., 1986). In the (Na^+,K^+)-ATPase (Shull et al., 1985) and in the more distantly related members of the family [see Solioz et al. (1987) and references cited therein], neither Cys residue is conserved. The lack of conservation in this region suggests that this area does not serve a critical functional role for the protein. Consistent with this, we have found virtually no functional effects arising from labeling of the Ca^{2+}-ATPase with IAF or IAEDANS.

An examination of the primary structure and predicted secondary structure of the Ca^{2+}-ATPase in the region of Cys_{674} and Cys_{670} suggests a mechanism to account for the higher reactivity of these 2 Cys residues over the approximately 14 Cys residues on the surface of the Ca^{2+}-ATPase (see the introduction). This region (residues 667–678) has been predicted to be either unstructured (Brandl et al., 1986) or α-helical (M. E. Kirtley, using the BIONET program, personal communication). A model based upon the α-helical prediction (Figure 5) shows the region to contain a cluster of Cys residues (670, 674, and 675) as well as a cluster of Arg residues (667, 671, 672, and 678). The cationic Arg residues would enhance sulfhydryl reactivity by decreasing the pK by stabilizing the reactive anionic thiolate form (Lundblad & Noyes, 1984). Such enhancement of reactivity has been noted, for example, in thioredoxin, where a Lys residue was shown to decrease the pK of a proximal Cys from 8.5 to 6.7 (Kallis & Holmgren, 1980). The Arg residues (Figure 5) would additionally enhance reactivity of the proximal Cys residues through charge attraction of the anionic IAF molecule. The lack of any detectable labeling of Cys_{675} might therefore be due to the influence of the neighboring Glu_{668} (Figure 5) to increase the sulfhydryl pK and to repel the anionic IAF.

Recent work from Kawakita's group (Saito-Nakatsuka et al., 1987; Yamashita & Kawakita, 1987) lends support to the above reactivity hypothesis. The primary labeling site for both the small, polar iodoacetamide and the anionic IAEDANS probes was identified as Cys_{674} [Yamashita & Kawakita, 1987; Cys_{674} has been confirmed as the IAEDANS site by Suzuki et al. (1987) using essentially the same labeling condition], as was found here for IAF. That no additional labeling of Cys_{670} was found by these authors may be due both to the stopping of the labeling reaction at 1 mol/mol of Ca^{2+}-ATPase (while reactions herein showed a single rapid phase of labeling to a final level of 2 mol/mol) as well as to the losses of 30–40% of the labels to the void fractions of the LH Sephadex separations (Yamashita & Kawakita, 1987). Interestingly, it was found further that N-ethylmaleimide exclusively labeled Cys_{344} and Cys_{364}, which are both in the A_1 tryptic fragment of Ca^{2+}-ATPase (Saito-Nakatsuka et al., 1987; Kawakita & Yamashita, 1987). That N-ethylmaleimide is considerably more apolar than charged sulfhydryl-directed reagents [see Reithmeier and MacLennan (1981) and references cited therein] and since the primary structure in the region of Cys_{344} and Cys_{364} is largely apolar (Brandl, et al., 1986) further suggest a significant contribution of polarity in steering different sulfhydryl-directed probes to different regions of the Ca^{2+}-ATPase molecule. Finally, it was found that the apolar N-(1-anilinonaphth-4-yl)maleimide probe labeled primarily the A_1 tryptic fragment (Miki et al., 1981) and it may well be attracted to the same apolar regions as is N-ethylmaleimide.

A tertiary model of the Ca^{2+}-ATPase has been proposed (Squier et al., 1987) on the basis of previous energy transfer experiments, secondary structure predictions (Brandl et al., 1986), and three-dimensional image reconstruction from crystals (Taylor et al., 1986). The main features of this model include (1) a cluster of transmembrane and amphipathic (stalk) helices and (2) a large lobe of helices and β-sheets arranged into a trigonal pattern, which connect to the top of this stalk and protrude into the cytoplasm. The entire enzyme can be divided by mild tryptic digestion into three fragments or putative domains: the phosphorylation domain (A_1 tryptic fragment), the transduction domain (A_2 tryptic fragment), and the nucleotide binding domain (B tryptic fragment). The present study confirms and further refines certain aspects of this model by the identification of the particular residues that are modified by IAF or IAEDANS.

For example, the distance between two probes (IAEDANS at the proximal Cys residues and FITC at the nucleotide site) on the B fragment is comparable to the size of the major axis of the entire cytoplasmic portion of the Ca^{2+}-ATPase. The procedure used in this study for the isolation of labeled peptides rules out the possibility that Cys_{670} and Cys_{674} are on transmembrane segments and thus confines its location to either

the stalk or the cytoplasmic portion of the B fragment. Location of these two Cys residues on the stalk region is unlikely by virtue of the absence of Cys residues modeled to be in this portion of the B fragment (Brandl et al., 1986). Therefore, the considerable distance from these two residues to the nucleotide probe FITC is consistent with the predicted secondary structure in as far as the carboxyl end of the B fragment is distal from the FITC-modified (Lys_{515}) amino end of this fragment and thus validates our previous conclusion that the B fragment must span the length of the entire cytoplasmic lobe of the Ca^{2+}-ATPase. Moreover, these measurements point out the large portion of the protein structure of this predicted nucleotide binding domain that is not devoted to the binding of nucleotide (the Cys_{670} and Cys_{674} region), as evidenced by the small nucleotide size (<20 Å) relative to the size of this domain.

On the other hand, the distance between IAEDANS and praseodymium bound in the high-affinity calcium sites is relatively short, i.e., 16–18 Å (Squier et al., 1986). The Ca^{2+} sites are associated with the A_2 fragment, but the exact residues involved in calcium binding have not been identified (Pick & Racker, 1979). The proximity of Cys residues 670 and 674 to these sites on the A_2 fragment confirms the proposed model, in which these three domains are folded onto one another such that the A_2 fragment is juxtaposed to the carboxyl end of the B fragment. These data are further consistent with the proposed high-affinity binding of calcium to glutamate residues on the stalk region (MacLennan et al., 1985); image reconstruction techniques have shown that the stalk region extends 16 Å between the bilayer and the cytoplasmic lobe (Taylor et al., 1986). However, these data do not rule out other locations on the A_2 fragment for the calcium sites.

A further aspect of the present study lies in its potential for future structural studies. We have now localized two residues in a nonfunctional region of the enzyme's structure that can be modified by fluorescence probes so as to approximate a single energy transfer locus. Therefore, these sites, in combination with other specific sites, can provide a means to monitor the conformation of the Ca^{2+}-ATPase throughout the transport cycle in a nonintrusive manner.

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Registry No. ATPase, 9000-83-3; IAF, 63368-54-7; Cys, 52-90-4.

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Thermal Denaturation of T4 Gene 32 Protein: Effects of Zinc Removal and Substitution†

Kathleen M. Keating,*,‡ Lily R. Ghosaini,§ David P. Giedroc,‡ Kenneth R. Williams,‡ Joseph E. Coleman,‡ and Julian M. Sturtevant‡,§

Department of Molecular Biophysics and Biochemistry and Department of Chemistry, Yale University, New Haven, Connecticut 06510

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ABSTRACT: Gene 32 protein (g32P), the single-stranded (ss) DNA binding protein from bacteriophage T4, is a zinc metalloprotein. The intrinsic zinc is one of the factors required for the protein to bind cooperatively to a ssDNA lattice. We have used differential scanning calorimetry to determine how the thermodynamic parameters characterizing the denaturation of g32P are affected by removal or substitution of the intrinsic zinc. Over a wide concentration range (1–10 mg/mL), the native Zn(II) protein unfolds at a t_m of 55 °C with an associated mean enthalpy change of 139 kcal mol^{-1}. Under the same conditions, the metal-free apoprotein denatures over a relatively broader temperature range centered at 49 °C, with a mean enthalpy change of 84 kcal mol^{-1}. Substitution of Zn(II) in g32P by either Cd(II) or Co(II) does not significantly change the enthalpy of denaturation but does affect the thermal stability of the protein. All metallo forms of g32P when bound to poly(dT) undergo highly cooperative denaturational transitions characterized by asymmetric differential scanning calorimetry peaks with increases in t_m of 4–5 °C compared to the unliganded metalloprotein. Removal of the metal ion from g32P significantly reduces the cooperativity of binding to poly(dT) [Giedroc, D. P., Keating, K. M., Williams, K. R., & Coleman, J. E. (1987) *Biochemistry 26*, 5251–5259], and presumably as a consequence of this, apo-g32P shows no change in either the shape or the midpoint of the thermal transition on binding to poly(dT). Thus, a single metal ion tetrahedrally coordinated to each g32P monomer provides the largest increase in the thermodynamic stability when the protein is cooperatively complexed with ssDNA. In accord with calorimetric data, we suggest that a change in the conformation or a reduction in conformational flux must occur as a direct consequence of the association of zinc with g32P which in turn enables highly cooperative binding of the protein to ssDNA.

Gene 32 of bacteriophage T4 codes for a nucleic acid binding protein (g32P)[1] that is required in stoichiometric amounts for DNA replication, repair, and recombination [for a review, see Chase and Williams (1986)]. Approximately one-third of the overall free energy of g32P binding to single-stranded nucleic acids derives from cooperative protein–protein interactions between adjacent g32P molecules bound to the nucleic acid lattice (Kelly et al., 1976). These cooperative g32P–g32P interactions are dependent in part upon an intact NH_2-terminal "B" region (defined here as residues 1–21). Proteolytic removal of the B region reduces the cooperativity parameters for binding ssDNA from about 10^3 to 1 (Spicer et al., 1979).

We have recently demonstrated that g32P contains an intrinsic Zn(II) ion (Giedroc et al., 1986). The visible absorption spectrum of the Co(II)-substituted protein is suggestive of a rather regular tetrahedral geometry about the metal ion while the presence of the two intense sulfur to Co(II) charge-transfer bands in the near-ultraviolet indicates coordination to sulfur. Mercurial titrations suggest the presence of three cysteine-S^- ligands to the Zn(II) ion (Giedroc et al., 1986). Oligonucleotide binding studies indicate that while the removal of the metal ion from g32P has relatively little effect on the binding affinity of g32P for a nucleic acid lattice containing only one binding site, it significantly decreases the binding affinity for a lattice having two contiguous binding sites due to a reduction in the cooperativity parameter by more than 2 orders of magnitude (Giedroc et al., 1987). As a result, apo-g32P is significantly less effective than native g32P at destabilizing a partially double-stranded polynucleotide such as poly[d(A-T)], completely covering a ssDNA lattice, and protecting ssDNA from endonuclease digestion (Giedroc et al., 1987; Keating et al., 1987). The presence of Zn(II) sta-

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‡ Department of Molecular Biophysics and Biochemistry.
§ Department of Chemistry.

[1] Abbreviations: g32P, gene 32 protein; ss, single stranded; PMBS, *p*-(hydroxymercuri)benzenesulfonate; DSC, differential scanning calorimetry; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TNG, 10 mM Tris-HCl, 0.2 M NaCl, and 5% v/v glycerol, pH 8.

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