Specific Labeling of the Bovine Heart Mitochondrial Phosphate Carrier with Fluorescein 5-Isothiocyanate

ROLES OF LYS\textsuperscript{185} AND PUTATIVE ADENINE NUCLEOTIDE RECOGNITION SITE IN PHOSPHATE TRANSPORT\textsuperscript{6}

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The amine/SH-modifying fluorescein 5-isothiocyanate (FITC) specifically labeled Lys\textsuperscript{185} in the putative membrane-spanning region of the phosphate carrier from both the cytosolic and matrix sides of bovine heart mitochondria at 0 °C and pH 7.2, and the labeling inhibited the phosphate transport. Nonmodifying fluorescein derivatives having similar structural features to those of ADP and ATP (Majima, E., Yamaguchi, N., Chuman, H., Shinohara, Y., Ishida, M., Goto, S., and Terada, H. (1998) Biochemistry 37, 424–432) inhibited the specific FITC labeling and phosphate transport, but the nonfluorescein phenylisothiocyanate did not inhibit FITC labeling, suggesting that there is a region recognizing the adenine nucleotides in the phosphate carrier and that this region is closely associated with the transport activity. The phosphate transport inhibitor pyridoxal 5'-phosphate inhibited the specific FITC labeling, possibly due to competitive modification of Lys\textsuperscript{185}. In addition, FITC inhibited the ADP transport and specific labeling of the ADP/ATP carrier with the fluorescein SH reagent eosin 5-maleimide. Based on these results, we discuss the structural features of the phosphate carrier in relation to its transport activity.

There are various solute carriers in the mitochondrial inner membrane to support ATP synthesis by oxidative phosphorylation. The 30-kDa solute carriers, consisting of a three-repeat structure containing a certain consensus sequence, are members of the mitochondrial solute carrier family (1). Of these, the ADP/ATP carrier mediating transport of ADP and ATP, the phosphate carrier mediating the symport of orthophosphate (P\textsubscript{i}) and H\textsuperscript{+}, and the type 1 uncoupling protein forming the short circuit of the proton current (2–4) have received considerable attention. These carriers take similar topologies of six membrane-spanning helices with three large hydrophilic loops facing the matrix, and their homodimers are thought to be their functional units (2, 3, 5, 6). However, their precise structural characteristics are not fully understood in relation to their transport functions. Because fluorescein derivatives have been thought to have similar structural features to those of adenine nucleotides (7, 8), they have been used as fluorescent probes in studies on the kinetics and conformational changes caused by their interactions with the adenine nucleotide binding sites of proteins such as ATPases (9–11), NAD(P)\textsuperscript{+}-dependent dehydrogenases (12, 13), and kinases (7, 8). In fact, we recently reported that the geometric and electronic structures of fluorescein analogs are very similar to those of ADP/ATP (14). In addition, we found that various fluorescein derivatives have high affinities to the ADP/ATP carrier in bovine heart mitochondria, and the binding leads to inhibition of the transport activity (4, 14, 15). Of the fluorescein analogs, the SH reagent eosin 5-maleimide (EMA)\textsuperscript{2} most significantly interacts with the ADP/ATP carrier; it quickly and specifically labels Cys\textsuperscript{159} in the second loop facing the matrix of the bovine heart mitochondrial carrier in competition with ADP, showing that the region around Cys\textsuperscript{159} is a major recognition and binding site of the adenine nucleotides (14–16). Accordingly, we studied the effect of the amine/SH-modifying fluorescein analog of fluorescein 5-isothiocyanate (FITC) on bovine heart mitochondria and the submitochondrial particles. Unexpectedly, it specifically labeled the 34-kDa mitochondrial phosphate carrier from both cytosolic and matrix sides at a physiological pH of 7.2. We further examined the effects of various fluorescein analogs (for chemical structures, see Structure I) on the FITC labeling and P\textsubscript{i} uptake of the phosphate carrier and those of FITC on EMA labeling and ADP uptake of the ADP/ATP carrier. Based on the results, we discuss the mode of binding of FITC in relation to the transport activity of the phosphate carrier.

EXPERIMENTAL PROCEDURES

Reagents—FITC (isofom I) and EMA were purchased from Molecular Probes (Eugene, OR). Fluorescein, eosin Y, and lysylendopeptidase were from Wako Pure Chemical Industries (Osaka, Japan), erythrosin B was from Fluka (Buchs, Germany), mersalyl was from Aldrich, and hydroxylapatite and AG 1-X8 were from Bio-Rad. Bongkrekic acid was a gift from Prof. Duine (Delft University of Technology).

Preparations of Bovine Heart Mitochondria and Submitochondrial Particles—Bovine heart mitochondria were prepared according to Smith (17). Submitochondrial particles containing 5 mM potassium phosphate were prepared by sonicating bovine heart mitochondria, as described previously (15). Mitochondria and submitochondrial particles were finally suspended in the standard assay medium consisting of 250 mM sucrose, 0.2 mM EDTA, and 10 mM Mops, pH 7.2 (S. E. medium). The amounts of proteins in mitochondria and submitochondrial parti-

\textsuperscript{1} The abbreviations used are: EMA, eosin 5-maleimide; Chex, 2-N-cyclohexyl-2-aminoethanesulfonic acid; DTT, dithiothreitol; FITC, fluorescein 5-isothiocyanate; PTP, phenylisothiocyanate; PIP, pyridoxal 5'-phosphate; HPLC, high performance liquid chromatography; Mes, 2-(N-morpholino)ethanesulfonic acid; Mops, 3-(N-morpholino)propane-sulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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Pi uptake was started by the addition of a final concentration of 1 mM potassium [32P]phosphate in S. E. medium and used for the assay of Pi transport. As a control, mitochondria were incubated for 3 min at 0 °C and then centrifuged. The sedimented mitochondria were washed with the solution used for electrophoresis consisting of 1% SDS, 1% dithiothreitol (DTT), and 25 mM Tris-HCl buffer (pH 6.8). Then, the samples (25 μg of protein) were subjected to SDS-PAGE on 12% polyacrylamide gel under reducing conditions according to Laemmli (18). The fluorescence intensities of protein bands labeled with FITC on the gel were determined with excitation at 500 nm, as described previously (15). For study of FITC labeling at various pH values, three Goed’s buffers were used; 40 mM Mes-NaOH for pH 5.0 and 6.0, 40 mM Mops-NaOH for pH 7.0 and 8.0, and 40 mM Chaps-NaOH for pH 9.0. For examination of the effects of test compounds on the labeling, the mitochondria or the particles (10 mg of protein/ml) were first incubated with a test compound for various periods, usually 10 min, at 0 or 37 °C and then incubated with FITC for 5 min at 0 °C in the dark, unless otherwise noted.

P<sub>i</sub> Uptake by the Phosphate Carrier—Freshly prepared mitochondria and submitochondrial particles (both 10 mg of protein/ml) suspended in S. E. medium were incubated with 200 μM FITC for 10 min at 0 °C in the dark to avoid possible damage of membrane proteins caused by singlet oxygen, which could be generated by fluorescein analogs in the light (14), and the labeling was terminated by 5-fold dilution of samples with the solution used for electrophoresis consisting of 1% SDS, 1% dithiothreitol (DTT), and 25 mM Tris-HCl buffer (pH 6.8). Then, the samples (25 μg of protein) were subjected to SDS-PAGE on 12% polyacrylamide gel under reducing conditions according to Laemmli (18). The fluorescence intensities of protein bands labeled with FITC on the gel were determined with excitation at 500 nm, as described previously (15). For study of FITC labeling at various pH values, three Goed’s buffers were used; 40 mM Mes-NaOH for pH 5.0 and 6.0, 40 mM Mops-NaOH for pH 7.0 and 8.0, and 40 mM Chaps-NaOH for pH 9.0. For examination of the effects of test compounds on the labeling, the mitochondria or the particles (10 mg of protein/ml) were first incubated with a test compound for various periods, usually 10 min, at 0 or 37 °C and then incubated with FITC for 5 min at 0 °C in the dark, unless otherwise noted.

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### Structure 1: Chemical structure of fluorescein derivative and other chemical modifiers.

Fluorescein was determined by a BCA protein assay kit (Pierce) in the presence of 1% SDS using bovine serum albumin as a standard.

#### Labeling with FITC—
For FITC labeling at pH 7.2, freshly prepared mitochondria and submitochondrial particles (both 10 mg of protein/ml) suspended in S. E. medium were incubated with 200 μM FITC for 10 min at 0 °C in the dark. After a 5-fold dilution, ADP transport was started with 20 μM [14C]ADP (specific radioactivity, 0.67 GBq/mmol), and ADP uptake was terminated after 10 s at 0 °C by the addition of 10 mM bongkrekic acid. The amount of [14C]ADP taken up by the particles was determined from the radioactivity of the incorporated [14C]ADP in an Aloka liquid scintillation counter, model LSC-3500 (15).

#### N-terminal Amino Acid Sequence Analysis—
The N-terminal amino acid sequences of the labeled protein and its peptide samples were determined with an HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA).

#### Determination of FITC-labeling Site—
Mitochondria and submitochondrial particles (both 10 mg of protein/ml) suspended in S. E. medium were incubated with 200 μM FITC for 20 min at 0 °C. After removing the free FITC by chromatography on a Sephadex G-50 column, the fluorescent eluates were solubilized with 5% Triton X-100 containing 0.5 mM NaCl, 0.5 mM EDTA, and 10 mM Mops (pH 7.2) for 10 min at 0 °C, and solubilized samples were applied to a hydroxylapatite column equilibrated with a solution consisting of 0.5% Triton X-100, 0.1 mM NaCl, 0.05 mM EDTA, and 10 mM Mops (pH 7.2). Proteins labeled with FITC in the flow-through fractions were precipitated with cold acetone at −20 °C, dissolved with 6 M guanidine HCl in 1 mM EDTA and 0.5 mM Tris-HCl buffer (pH 8.5), and treated with DTT (0.67 μmol/mg of protein) for 2 h at 37 °C to reduce all the cysteine residues. The cysteine residues were then carboxamidomethylated with freshly prepared iodoacetamide (1.41 μmol/mg of protein) for 30 min at room temperature in the dark. The alkylation of proteins was terminated with DTT (4.2 μmol/mg of protein), and the sample solution was applied to a column of G4000SWg<sub>4</sub> (7.8 × 300 mm, Tosoh, Tokyo) equilibrated with 0.05% trifluoroacetic acid containing 63% acetonitrile and eluted at a flow rate of 0.5 ml/min. The protein labeled with FITC was detected by monitoring the absorbance at 280 nm and fluorescent intensity at 510 nm by excitation with 450 nm. The fluorescent fractions were pooled and concentrated with a Speed Vac concentrator (Savant, New York). After dilution of the FITC-labeled protein 5-fold with 200 mM Tris-HCl buffer (pH 8.0), it was digested with lysylendopeptidase (2% w/w) for 20 h at 35 °C. The digest was diluted 4-fold with 7 M guanidine HCl, and peptide fragments were separated by reversed-phase HPLC on a TSK gel ODS-120T column (4.6 × 150 mm, Tosoh) with linear gradients of acetonitrile at 0–12% for 5 min, 12–52% for 80 min, and 52–90% for 5 min in 0.05% trifluoroacetic acid at a flow rate of 1 ml/min monitored with absorbance at 210 nm and fluorescence at 510 nm excited at 450 nm. The eluate was collected at intervals of 1 min.

#### RESULTS

#### Effect of FITC on Mitochondrial Proteins—
First we examined the labeling of mitochondrial proteins with fluorescein analogs, the SH reagent EMA, and amine/SH-modifier FITC. Freshly prepared bovine heart mitochondria and their submitochondrial particles were incubated with 200 μM EMA for 30 s at 0 °C and pH 7.2 in the dark to avoid possible production of singlet oxygen. Alternatively, suspensions of mitochondria and submitochondrial particles were incubated with 200 μM FITC for 10 min at 0 °C in the dark. Then these samples were subjected to SDS-PAGE. As shown in Fig. 1, EMA predominately labeled the 30-kDa ADP/ATP carrier (band AAC) in the submitochondrial particles but not mitochondria, as we reported previously (15). EMA also labeled the 34-kDa protein slightly. In contrast, FITC did not label the ADP/ATP carrier, but it selectively labeled the 34-kDa protein both in mitochondria and submitochondrial particles (Fig. 1). Because membrane-impermeable EMA labeled the ADP/ATP carrier only from the matrix side (15, 20), the labeling of the 34-kDa protein by FITC from both the cytosolic and matrix sides was not due to damage of the mitochondrial membrane.

Next we incubated mitochondria and the particles with various concentrations of FITC for 10 min at 0 °C and pH 7.2. After termination of the labeling, progress of the labeling was assayed from the intensity of the fluorescent band on SDS-PAGE. As shown in Fig. 2A, the labeling of the 34-kDa protein by FITC in both membrane systems increased with increase in the FITC concentration.

#### Effect of FITC on EMA Labeling and Transport Activity of the ADP/ATP Carrier—
The effect of FITC on EMA labeling of the ADP/ATP carrier was examined by incubation of the particles (10 mg of protein/ml) with various concentrations of FITC in S. E. medium at 0 °C for 10 min in the dark. The particles (2 mg protein/ml) were then incubated with 20 μM EMA for 30 s, and labeling was terminated with excess DTT (10 μmol/ml of protein). The particles were subjected to 12% SDS-PAGE, and the fluorescent intensity of the 30-kDa band due to the labeled ADP/ATP carrier was determined in a Shimadzu chromatoscan-
The time course of the labeling was examined with 200 μM FITC for 10 min at pH 7.2 in the dark. After termination of the labeling as described in the legend of Fig. 2B, the greater the labeling of the particles than mitochondria should be due to the higher content of the 34-kDa protein in the particles, which contain only mitochondrial membrane proteins, showing that FITC specifically labeled the 34-kDa protein to a similar extent from both the cytosolic and matrix sides at 0 °C at a physiological pH of 7.2.

Identification of the 34-kDa Protein Labeled by FITC—For identification of this 34-kDa protein, mitochondria were incubated with 200 μM FITC at pH 7.2 and 0 °C for 20 min, when FITC labeling almost attained the plateau level (see Fig. 2B). After solubilization of the treated mitochondria with Triton X-100, samples were applied to a column of hydroxylapatite gel. The labeled protein in flow-through fractions was denatured with guanidine HCl, and the cysteine residues were reduced with DTT and carboxamidomethylated with iodoacetamide. The labeled protein was isolated by gel filtration chromatography. After SDS-PAGE, the fluorescent band was transferred to a polyvinylidene difluoride membrane and then subjected to amino acid sequence analysis.

The N-terminal sequence of 51 amino acid residues was determined as AVEEYQSCDYGRFFILCGLGIGISQCGKIBXQEQYSCDYGRGRFFILCGLGGIISCGTT-HTALVPLDLVHKCRMVQDPQKY. Because this sequence was the same as the N-terminal sequence of the bovine heart mitochondrial phosphate carrier (21–23), the phosphate carrier was concluded to be specifically labeled with FITC at 0 °C. FITC did not label the N-terminal α-amino group of Ala1, the ε-amino groups of Lys41 and Lys42, or the SH groups of Cys19, Cys27, and Cys42 in this N-terminal region of the carrier at pH 7.2. However, it is not certain whether FITC actually did label the above cysteine residues because it is possible that treatment of the phosphate carrier with DTT released the labeled FITC, as described later.

Effect of FITC on P_i Uptake via the Phosphate Carrier—Next, we examined the effect of FITC labeling on P_i uptake mediated by the phosphate carrier. For this, we incubated mitochondria with various concentrations of FITC for 10 min at pH 7.2 and 0 °C and started P_i transport by the addition of [32P]Pi. After 30 s, P_i uptake was terminated with the phosphate transport inhibitor mersalyl, and the amount of P_i incorporated into the mitochondria was determined. As shown in Fig. 3, FITC inhibited P_i uptake depending on the FITC concentration. The transport in the mitochondria was almost completely inhibited by 200 μM FITC, and the concentration necessary for 50% inhibition (IC_{50}) was about 60 μM. A similar inhibitory effect was observed with the particles (Fig. 3).

Because FITC labels the deprotonated SH group and amino group (24), it was necessary to determine whether inhibition of P_i uptake via the phosphate carrier by FITC was due to the labeling of cysteine and/or lysine residues in the carrier. We examined the effect of DTT on P_i uptake by mitochondria labeled with FITC. If the P_i transport inhibition was due to labeling of a cysteine residue(s) in the carrier, DTT treatment should cause loss of P_i transport inhibition (25). We incubated FITC-treated mitochondria with 10 mM DTT for 10 min at 0 °C, then removed the added DTT by washing the mitochondria and examined their P_i transport activity. We confirmed that DTT completely abolished the inhibited P_i uptake caused by SH-modifying reagents such as mersalyl (data not shown). As
solved with 6 M guanidine HCl and treated with DTT to reduce precipitate proteins. Then, the precipitated proteins were digested with the lysine-specific proteinase lysylendopeptidase, and the cysteine residues were carboxamidomethylated samples were subjected to gel filtration chromatography to isolate the FITC-labeled phosphate carrier. The fluorescent peptide fluorescent peak was observed besides the FITC-labeled fluorescent peptide peak in the reversed-phase HPLC on digestion of the FITC-labeled Ca$^{2+}$-ATPase with trypsin (26).

The fluorescent fraction producing peak 2 from submitochondrial particles was subjected to amino acid sequence analysis, and the results are summarized in Table I. This peptide consisted of 29 amino acid residues, but the 16th residue could not be determined due to modification with FITC. According to the sequence of the bovine heart mitochondrial phosphate carrier (21, 22), the determined sequence was found to correspond to that from Gly$^{170}$ to Lys$^{185}$. Therefore, the 16th residue of the peptide was Lys$^{185}$, located in the putative fourth transmembrane segment (27). Similarly, Lys$^{185}$ of the phosphate carrier in mitochondria was determined to be modified by FITC. Therefore, the hydrophilic and anionic FITC specifically labeled the same lysine residue from both the cytosolic and matrix side.

**Effect of pH on FITC Labeling**—It is well known that acylation of amines by isothiocyanates is significantly pH-dependent and that FITC modification of lysine residues proceeds well at alkaline pH values, in which lysine residues are deprotonated (28). We examined the labeling of the phosphate carrier with 200 $\mu$M FITC at 0 °C at various pH values between pH 5.0 and 9.0. The results of SDS-PAGE showed that FITC specifically labeled the phosphate carrier in mitochondria and the particles at various pH values examined, and the labeling was significant at pH 6.0 and 7.0 (Fig. 5). It is noteworthy that FITC was not essentially reactive with the phosphate carrier at pH 9.0. Fig. 6 shows the results of pH-dependent FITC labeling determined from the intensities of the fluorescent bands shown in Fig. 5. FITC labeling increased with increase in pH, attaining a maximum level at pH 7.0, and then decreased with further increase in pH in both mitochondria and the particles.

It was surprising that the affinity of FITC to the phosphate carrier at pH 8.0 was remarkably less than that at pH 7.0 and that FITC was not reactive at pH 9.0, although FITC labeling was expected to be more favorable in the alkaline pH region than in the neutral region. When the labeling was examined at higher temperatures such as 37 °C, FITC labeled the ADP/ATP carrier in addition to the phosphate carrier at pH 7.2, and it labeled various mitochondrial proteins nonspecifically, including these two proteins at pH 9.0 (data not shown). Because the pH dependence of the FITC labeling was similar to that of $P_i$ transport via the phosphate carrier (29), the binding of FITC to the carrier is closely associated with its transport function.

**Effects of Pyridoxal 5′-Phosphate and Phenylosiiothiocyanate on the Phosphate Carrier**—The phosphate analog pyridoxal 5′-phosphate (PLP), which modifies lysine residues in proteins by forming a Schiff base (30), has been used as an inhibitor of the phosphate carrier (31). We examined the effects of various concentrations of PLP on the labeling with 200 $\mu$M FITC and the $P_i$ transport activity of mitochondria at pH 7.2 and 0 °C. As shown in Fig. 7, the FITC labeling of the carrier was decreased with increases in the PLP concentration, and labeling was almost completely inhibited by 20 mM PLP.

![Fig. 3. Concentration-dependent effect of FITC on $P_i$ uptake by mitochondria and submitochondrial particles.](http://www.jbc.org/)
PLP inhibited the \( P \) uptake in a similar manner to its inhibition of FITC labeling. However, the inhibitory effect of PLP was not complete, and about 30% of the transport activity was consistently preserved at 15 mM and above (Fig. 7). This could be due to reverse reaction of the Schiff base by dilution of PLP in the assay of transport activity (32). The PLP concentration requiring for maximum inhibition of \( P \) transport was consistent with the result in Stappen and Krämer (31). A similar inhibitory effect of PLP on the FITC labeling was observed with

| Cycle | Phenylthiohydantoin-derivative pmol | Cycle | Phenylthiohydantoin-derivative pmol |
|-------|-----------------------------------|-------|-----------------------------------|
| 1     | Gly (56.9)                        | 16    | ND\(^a\)                           |
| 2     | Val (54.9)                        | 17    | Phe (9.6)                          |
| 3     | Ala (63.1)                        | 18    | Ala (9.9)                          |
| 4     | Pro (15.1)                        | 19    | Cys (NQ)\(^b\)                    |
| 5     | Leu (20.9)                        | 20    | Phe (13.1)                         |
| 6     | Trp (17.2)                        | 21    | Glu (7.2)                          |
| 7     | Met (18.4)                        | 22    | Arg (6.1)                          |
| 8     | Arg (9.4)                         | 23    | Thr (4.7)                          |
| 9     | Gln (14.7)                        | 24    | Val (6.8)                          |
| 10    | Ile (8.4)                         | 25    | Glu (9.0)                          |
| 11    | Pro (8.5)                         | 26    | Ala (7.2)                          |
| 12    | Tyr (12.2)                        | 27    | Leu (6.0)                          |
| 13    | Thr (6.1)                         | 28    | Tyr (5.0)                          |
| 14    | Met (10.0)                        | 29    | Lys (2.5)                          |
| 15    | Met (18.7)                        |       |                                   |

The phosphate carrier purified from submitochondrial particles treated with FITC was denatured with guanidine-HCl, and its cysteine residues were carboxamidomethylated with iodoacetamide after reduction with dithiothreitol. Then the FITC-labeled carrier was digested with lysylendopeptidase, and the peptide fragments thus obtained were separated by reversed-phase HPLC on an ODS-120T column in a linear gradient of acetonitrile at a flow rate of 1 ml/min. For details, see “Experimental Procedures.” The elution profile was monitored as optical absorbance at 210 nm and fluorescence intensity at 510 nm excited at 450 nm. Elution profiles A and B (top and bottom chromatograms) show peptide fragments from the particles monitored by optical absorbance and fluorescence, respectively. In B, \(+\)LEP and \(-\)LEP are elution profiles of peptide fragments with and without treatment with lysylendopeptidase, respectively.

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**Fig. 4.** Reversed-phase HPLC profiles of peptides from FITC-labeled protein digested with lysylendopeptidase. Mitochondria (Mito) or submitochondrial particles (SMP) at 10 mg of protein/ml were incubated with 200 \( \mu \)M FITC at 0 °C for 20 min at pH 7.2 in the dark. After isolation, the FITC-labeled protein was incubated with lysylendopeptidase (LEP) at pH 8.0 for 20 h at 35 °C. The peptide fragments thus obtained were separated by reversed-phase HPLC on an ODS-120T column in a linear gradient of acetonitrile at a flow rate of 1 ml/min. For details, see “Experimental Procedures.” The elution profile was monitored as optical absorbance at 210 nm and fluorescence intensity at 510 nm excited at 450 nm. Elution profiles A and B (top and bottom chromatograms) show peptide fragments from the particles monitored by optical absorbance and fluorescence, respectively. In B, \(+\)LEP and \(-\)LEP are elution profiles of peptide fragments with and without treatment with lysylendopeptidase, respectively.

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**Fig. 5.** The effect of pH on the FITC labeling of proteins in bovine heart mitochondria (A) and their submitochondrial particles (B). The suspensions of bovine heart mitochondria (Mito) and the particles (SMP), each at 10 mg of protein/ml, were incubated with 200 \( \mu \)M FITC for 10 min at 0 °C at various pH values in the dark. After termination of the labeling, samples were subjected to SDS-PAGE. Experimental conditions were essentially as for Fig. 1.
the particles (data not shown). These results showed that PLP inhibited the P<sub>i</sub> transport possibly by binding to Lys<sup>185</sup> of the carrier in competition with FITC, although its affinity was much less than that of FITC. Accordingly, FITC was suggested to bind to the phosphate binding site of the carrier more efficiently than PLP.

The lysine-specific reagent phenylisothiocyanate (PITC) does not contain a fluorescein moiety (for chemical structure, see Structure I), and it is known to inhibit the P<sub>i</sub> transport in bovine heart mitochondria, but it is effective at the very high concentration of 5 mM (25). In addition, the inhibition is more favorable at pH 9.0 than at neutral pH (25). Because it is possible that the hydrophobic PITC preferentially modifies lysine residues in the membrane region of the phosphate carrier, we examined its effect on FITC labeling of the phosphate carrier. Mitochondria and submitochondrial particles that had been preincubated with various concentrations of PITC at pH 7.2 and 37 °C were incubated with 200 μM FITC at 0 °C, and the effect of PITC on FITC labeling was determined from the fluorescent band intensity on SDS-PAGE. As shown in Fig. 7, FITC labeling of the carrier in mitochondria was not affected at all by pretreatment with PITC at up to 20 mM. Even at 50 mM, PITC was ineffective in preventing FITC labeling of the carrier of mitochondria and submitochondrial particles (data not shown). These results clearly showed that PITC did not modify Lys<sup>185</sup>, unlike FITC, and thus, the fluorescein moiety of FITC is responsible for specific labeling of Lys<sup>185</sup>.

Effects of Fluorescein and Its Analogs on the Phosphate Carrier—We next examined the effects of fluorescein and its derivatives such as eosin Y and erythrosin B (for chemical structures, see Structure I) on the FITC labeling of the phosphate carrier and P<sub>i</sub> uptake. These analogs all possess a fluorescein moiety like EMA and FITC, and they interact noncovalently with the adenine nucleotide binding site of the ADP/ATP carrier (14). We incubated the mitochondria with these fluoresceins at pH 7.2 and 0 °C and used samples for examination of the effects on labeling of the phosphate carrier with 200 μM FITC and P<sub>i</sub> uptake. The effects on FITC labeling was determined from the intensity of the fluorescent band labeled with FITC on SDS-PAGE. As shown in Fig. 8A, the fluorescein derivatives inhibited FITC labeling in mitochondria depending on their concentrations. Erythrosin B was the most effective, fluorescein was slightly effective, and eosin Y was intermediate. The 50% inhibitory concentrations (IC<sub>50</sub>) of erythrosin B and eosin Y were 0.25 and 1.1 mM, respectively. Similar effects were observed with the particles (data not shown).

These fluoresceins inhibited P<sub>i</sub> uptake by mitochondria, as shown in Fig. 8B. The magnitudes of their inhibitory effects were in the order erythrosin B, eosin Y, and fluorescein, as observed with their inhibitions of FITC labeling, but their inhibitory concentrations were much less than those observed with FITC labeling due to competition of the noncovalent binding of P<sub>i</sub> and fluoresceins with the phosphate carrier. Namely, the IC<sub>50</sub> values of erythrosin B and eosin Y were 37 and 125 μM.
**FIG. 9. Effects of FITC on EMA labeling and ADP uptake.** Sub-mitochondrial particles (10 mg of protein/ml) were incubated with various concentrations of FITC at 0 °C and pH 7.2 for 10 min in the dark. After diluting the suspension to 2 mg of protein/ml, an aliquot of the sample suspension was treated with 20 μM EMA at 0 °C for 30 s, and the fluorescent intensity of the band labeled with EMA on SDS-PAGE was determined. The remaining part of the suspension was subjected to the assay of ADP uptake at 0 °C for 10 s. Experimental conditions were essentially as for Fig. 4, but 20 μM [14C]ADP was used for the transport substrate. The values of concentrations of FITC on the abscissa are those after dilution of the particle suspensions. The values (± S.D.) are means for three separate experiments. In the absence of FITC, ADP uptake was 720 pmol/min/mg of protein by mitochondria.

respectively, and that of fluorescein was more than 1 mM. The inhibitory effects of fluorescein and its analogs on FITC labeling and P uptake depended on their hydrophobicities, as observed with their effects on the ADP/ATP carrier (14). Because these fluorescein analogs interact noncovalently with the adenine nucleotide binding site (14), their inhibitions of FITC labeling and P uptake suggested that there is a region recognizing the adenine nucleotide moiety in the phosphate carrier and that this moiety is associated with P transport.

**Effect of FITC on the ADP/ATP Carrier—**Because various fluorescein analogs interact with the binding site of adenine nucleotides of the ADP/ATP carrier (4, 14), we examined the effect of FITC on the ADP/ATP carrier. The particles preincubated with various concentrations of FITC at pH 7.2 and 0 °C were further incubated with 20 μM EMA, which predominantly labels Cys159 of the bovine heart mitochondrial ADP/ATP carrier only from the matrix side (15). After incubation, the particles were subjected to SDS-PAGE, and the effect of FITC was determined from the intensity of the fluorescent band due to the labeled ADP/ATP carrier with EMA. As shown in Fig. 9, FITC inhibited the EMA labeling of the ADP/ATP carrier, and its effect became greater with increase in its concentration.

ADP transport was started by the addition of [14C]ADP to the particles preincubated with FITC at 0 °C and pH 7.2, and the amount of [14C]ADP incorporated into the particles for 10 s was determined. As shown in Fig. 9, FITC inhibited ADP uptake by the particles in a concentration-dependent manner, and its effect on ADP uptake was more significant than that on EMA labeling due to its noncovalent competition with ADP. These results suggested that the fluorescent moiety of FITC interacted with the nucleotide binding site of the ADP/ATP carrier.

**DISCUSSION**

In this study, we examined the effects of the amine/SH-reactive fluorescein analog FITC on bovine heart mitochondria and their particles. At a physiological pH and 0 °C, it specifically labeled Lys185 in the putative fourth transmembrane segment of the phosphate carrier from both the cytosolic and matrix sides, and the labeling inhibited P uptake mediated by the phosphate carrier. The same P transport inhibitions by FITC with and without DTT treatment clearly showed that the possible labeling of cysteine residues with FITC (24) was not associated with P transport inhibition by FITC. This conclusion is supported by the finding that replacement by Ala or Arg of Lys187 of the yeast carrier, which corresponds to Lys185 of the bovine carrier, resulted in complete loss of the transport activity (33).

It is noteworthy that besides labeling Lys185 of the phosphate carrier, FITC inhibited the specific labeling of Lys159 of the ADP/ATP carrier with the fluorescein SH reagent EMA and ADP transport across mitochondrial inner membrane. These results showed that FITC also interacted with the ADP/ATP carrier. Because the geometric and electronic structural features of the fluorescein moiety are very similar to those of ADP/ATP (14), fluorescein and its analogs can be used as efficient probes for characterization of the adenine nucleotide binding region (7–14). Accordingly, it is suggested that there is an adenine nucleotide recognition site in the phosphate carrier, as in the ADP/ATP carrier. In fact, fluorescein and its analogs such as eosin Y and erythrosin B prevented the specific FITC labeling of Lys185 and inhibited P transport.

Because the pH dependence of the specific labeling of Lys185 with FITC was similar to that of P transport via the phosphate carrier (29) and because the phosphate analog PLP was found to inhibit specific FITC labeling and P transport equally from the cytosolic and matrix sides, Lys185 should be closely associated with the transport activity of the phosphate carrier. The importance of this residue is suggested from the fact that the lysine residue corresponding to Lys185 is conserved completely in all the nine mitochondrial phosphate carriers reported to date (1, 34).

Alkaline pH conditions are advantageous for nucleophilic attack of the isothiocyanate moiety on the deprotonated neutral form of the e-amino group of lysine residues (28). FITC labels a certain lysine residue of various proteins such as Na+,K+-ATPase (35, 36) and H+-ATPase (37) at pH 9.2 and 25 °C, and the labeling increases at higher pH values (36, 37). We found that FITC nonspecifically labeled various membrane proteins in bovine heart mitochondria when labeling was performed at pH 9.0 and 37 °C. However, FITC specifically labeled Lys185 of the phosphate carrier at a physiological pH and lower temperature of 0 °C. No labeling of proteins other than the phosphate carrier was observed under such conditions, although there are versatile membrane proteins in the mitochondrial membranes. In addition, it is noteworthy that FITC specifically recognized Lys185 among 24 lysine residues of the phosphate carrier under these conditions. This should be mainly due to the fact that Lys185 in the transmembrane is deprotonated at a physiological pH, whereas others are protonated or embedded in the membrane.

Because Lys185 is located in the inner half of the putative fourth membrane-spanning region, this residue could constitute the path for the transport of P. It is noteworthy that polar FITC accessed to Lys185 in transmembrane segment equally from both the cytosolic and matrix sides. Possibly, Lys185 functions as an acceptor/donor of H+, which is transported simultaneously with P in their proton symport. The transport of H+ through the membrane has been well studied with bacteriorhodopsin (38, 39). In this case, H+ is transported by interconversion of protonation/deprotonation of Lys216/retinal located in the center of the transmembrane segment. Similarly, ionizable amino acid residues other than Lys185 are necessary for conducting proton transfer from/to Lys185 in the phosphate carrier, like Asp196 and Glu204 in bacteriorhodopsin. In this...
respect, it is noteworthy that His32, Glu126, and Glu137 in the membrane-spanning region of the yeast phosphate carrier were suggested to be related with proton transfer (40).

Because both the phosphate carrier and the ADP/ATP carrier are mitochondrial solute carriers possibly evolving from the same ancestor protein (1), it is not surprising that there could be an adenine nucleotide recognition site in the phosphate carrier. The important role of the nucleotide recognition site in P1 transport is supported by the finding that nonamine-reactive fluorescein and its analogs having similar structural features to those of ADP/ATP inhibited P1 transport efficiently in a manner similar to their inhibition of adenine nucleotide transport through the ADP/ATP carrier (14). In addition, it is reported that P1 transport is competitively inhibited by ATP (29).

It is thought that the region around Cys159 in the second loop facing the matrix space constitutes the nucleotide binding site in the bovine heart mitochondrial ADP/ATP carrier (14, 16). Therefore, it is possible that the nucleotide recognition site of the phosphate carrier is located similarly in the second loop facing the matrix side, and this region should be located geometrically close to Lys185 in the putative fourth transmembrane segment. The fluorescein moiety of FITC should first interact with the putative nucleotide recognition site of the phosphate carrier, and then the isothiocyanate moiety of the bound FITC should label Lys185. In fact, amine-reactive PITC, which does not have a fluorescein moiety, did not label Lys185 at all, and the higher concentration of the phosphate analog PLP was necessary for inhibition of FITC labeling and P1 transport by its binding with Lys185, showing that the binding of FITC with the nucleotide binding site of the phosphate carrier before the modification of Lys185 is necessary for its specific and efficient labeling of Lys185 and subsequent P1 transport inhibition.

At present, it is not clear how the nucleotide recognition site takes part in P1 transport. However, we think that this site functions as a regulator of P1 transport. We found that the cooperative conformational changes in all the three loops facing the matrix side of the ADP/ATP carrier take place upon binding of the transport substrates ADP/ATP to their major recognition site in the second loop (4, 14, 16). By these conformational changes, the specific and efficient transport of ADP/ATP through the transport path is achieved (4, 5). A similar transport mechanism could operate in the transport activity of the phosphate carrier. Studies on the role of the adenine nucleotide recognition site in P1 transport are under way.

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Specific Labeling of the Bovine Heart Mitochondrial Phosphate Carrier with Fluorescein 5-Isothiocyanate: ROLES OF LYS185 AND PUTATIVE ADENINE NUCLEOTIDE RECOGNITION SITE IN PHOSPHATE TRANSPORT

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