Fluorescent and Spin Label Probes of the Environments of the Sulfhydryl Groups of Porcine Muscle Adenylate Kinase*

(Received for publication, July 1, 1974)

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SUMMARY

The environments of the two sulfhydryl groups of porcine muscle adenylate kinase have been investigated by chemical modification reactions. The results indicate that the environments of the two —SH groups of porcine muscle adenylate kinase are markedly different and that substrates induce conformational changes in the enzyme in the region of the sulfhydryl groups.

The fluorogenic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) reacts specifically with the —SH groups of the enzyme at pH 7.9. One thiol group reacts with NBD-chloride approximately 40-fold faster than the other one, and the fast reacting group has been identified as Cys-25 in the amino acid sequence. The similarity of the rate of the more slowly reacting Cys-187 with NBD-chloride to that of glutathione with the same reagent is consistent with its location on the surface of the enzyme as determined by x-ray crystallography structure. The fast reacting Cys-25 in the interior of the structure can be approached by compounds such as NBD-chloride via a cleft. Reaction of Cys-25, presumably located close to the catalytic center, leads to complete inactivation of the enzyme. Substrates such as ATP, MgATP, and ADP which bind to the triphosphate subsite of the enzyme decrease the rate of reaction of Cys-25 by factors up to 3.5 but have only a small effect (~10%) on the reactivity of Cys-187. AMP, however, has a pronounced effect on the reactivity of Cys-187, the slowly reacting group. The multisubstrate analogue P1,P5-di-(adenosine-5')pentaphosphate (Ap5A) decreases the rate of reaction of the fast reacting thiol group by a factor of 300.

The behavior of Cys-25 toward NBD-chloride, i.e. superreactivity in the absence of Ap5A and slow reactivity in the presence of the multisubstrate inhibitor, was characteristic for both porcine and carp adenylate kinase. In the presence of Ap-A adenylate kinase can be selectively modified at Cys-187; the introduction of the fluorescent NBD group at this position has no effect on enzymatic activity.

A slow transfer of the NBD group occurs from the thiol groups to the ε-amino group of Lys-31. The transfer reaction is further evidence that the structure of adenylate kinase in dilute solution is similar to that of the crystalline enzyme since the x-ray data have shown that the sulfur of Cys-187 and the ε-nitrogen of Lys-31 are less than 4 Å apart. The strongly fluorescent NBD-NH-enzyme possesses full activity and binds substrates as well as the unmodified enzyme.

The —SH groups have also been modified with a spin label derivative of iodoacetamide. Two superimposed spectra were observed for the modified enzyme, corresponding to a strongly immobilized and a weakly immobilized bound spin label. Addition of substrates (ATP, MgATP, GTP, MgGTP, and AMP) caused large changes in the electron paramagnetic resonance spectrum of the spin-labeled enzyme, mainly in that part of the spectrum arising from the weakly immobilized spin label. The effect of MnATP on the spin-labeled spectrum indicated that the manganese is approximately 20 Å from the enzyme-bound nitroxide group which gives rise to the immobilized spectrum.

Adenylate kinase (ATP:AMP-phosphotransferase, EC 2.7.4.3) appears to be one of the most suitable enzymes for relating structure and function of phosphoryl-transferring enzymes. The muscle enzyme is obtainable in a crystalline form and consists of a single polypeptide chain of molecular weight 21,700 (2, 3). Recently the amino acid sequence and a 3-A resolution x-ray crystallographic structure of the enzyme from porcine muscle have become available (4). In addition the enzyme is ideally suited to investigation by high resolution nuclear magnetic resonance spectroscopy, since there are no tryptophan and only two histidine residues in the molecule. A preliminary re-
port of magnetic resonance work on the enzyme has been published (5).

Relatively little work has been done to establish the roles of amino acid residues in the enzyme. Previous work has suggested that the two cysteine groups behave similarly in their reaction toward iodoacetate and 1-fluoro-2,4-dinitrobenzene (6) and that the cysteine residues are not directly involved in the catalysis, since derivatives of the enzyme can be prepared in which both sulphydryl groups have been reacted with mercurials without complete loss of activity (4, 7). Other studies have indicated that a methionine (8) and a histidine (9) may be involved in the enzyme mechanism.

In this paper we present some results of investigations into the environments of the cysteine groups of the enzyme. The first part deals with the use of a fluorogenic reagent, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, which reacts specifically with —SH groups near neutral pH to form a derivative with characteristic absorption and fluorescence properties (10). The absorption band facilitates a detailed analysis of the kinetics of the reaction which clearly indicates that the two cysteine residues have markedly different environments. The second part deals with spin-labeling experiments. Both probes afford evidence for substrate-induced conformational changes in the enzyme.

MATERIALS AND METHODS

Carp muscle adenylate kinase was prepared according to Noda et al. (11). Since carp adenylate kinase contains only one thiol group (11), the concentration of cysteine (12) was used for protein determination. The isolation of porcine muscle adenylate kinase has been described previously (3). Some of the porcine enzyme used in this work was a generous gift of Dr. L. Noda of Dartmouth University and Dr. G. G. McDonald of the University of Pennsylvania. The specific activity of the enzyme was 2,000 ± 100 units mg⁻¹ (assayed by the coupled hexokinase-phosphate assay (6)). The coupled assay with pyruvate kinase and lactate dehydrogenase was also used. In the latter assay, the solutions contained 1 mM AMP, 1 mM ATP, 1 mM P-enolpyruvate, 1 mM MgSO₄, 130 mM KCl, 50 mM K⁺-Hepes at pH 7.9, 25 pg of pyruvate kinase, 1 mg of lactate dehydrogenase; the reaction was initiated by the addition of 0.1 μg of adenylate kinase. The concentrations of solutions of porcine adenylate kinase were determined spectrophotometrically at 279 nm with the use of a molar absorbance of 11,700 M⁻¹ cm⁻¹ (11).

NBD-chloride was synthesized as described previously (10). Reaction with adenylate kinase was carried out by addition of a small aliquot of a solution of NBD-chloride in ethanol. The stoichiometry of the reaction was confirmed by titration of the —SH content of the enzyme before and after reaction with NBD-chloride, with the use of the known molar absorbance at 420 nm of the —S-NBD product (10). There was no evidence from the spectrum of the product for significant reaction of any other group in the enzyme. It was found that the precipitation of the enzyme derivatives could be retarded to a small extent by carrying out reactions at 0°, and by inclusion of salt (e.g. 0.2 M tetramethylammonium chloride) in the reaction medium.

Reaction of Adenylate Kinase with NBD-chloride

Stoichiometry of Reaction—Near neutral pH, NBD-chloride is almost totally specific for —SH groups (10). Reaction with other types of amino acid side chains (e.g. the ε-amino group of lysine and the phenolic hydroxyl group of tyrosine) have been reported under more drastic conditions (14, 15), but the products with these side chains can be easily distinguished from that with —SH groups on the basis of the spectrum of the product (10, 14, 15).

Under the conditions employed (50 mM KH₂PO₄ buffer at pH 7.9), NBD-chloride was found to react stoichiometrically with the two —SH groups of porcine adenylate kinase, with the use of the known molar absorbance at 420 nm of the —S-NBD product (10). There was no evidence from the spectrum of the product for significant reaction of any other group in the enzyme with NBD-chloride. The stoichiometry of the reaction was confirmed by titration of the —SH content of the enzyme before and after reaction with NBD-chloride, with the use of 5,5'-dithiobis-(2-nitrobenzoic acid) (12).

Kinetic Analysis of Reaction—The reaction with NBD-chloride was carried out under pseudo first order conditions to facilitate the kinetic analysis (16). The data for the reaction of 375 μM NBD-chloride with 10 μM adenylate kinase are shown in the form of a semilogarithmic plot in Fig. 1A. It is clear that the reaction is biphasic, and from the later, linear, portion of the plot it is possible to calculate the pseudo first order rate constant of the "slow" phase of the reaction. Division by the concentration of NBD-chloride gives the second order rate constant. The data for the reaction of 375 μM NBD-chloride with 10 μM adenylate kinase are shown in the form of a semilogarithmic plot in Fig. 1A.
A, semilogarithmic plot of $A_{\text{fast}}/A_{\text{slow}} - A_{\text{t}}$ as a function of time for the reaction of NBD-chloride (375 µM) with adenylate kinase (10 µM) in 50 mM K+-Hepes, pH 7.9, at 20°. $A_{\text{t}}$ represents the absorbance change at 420 nm after time $t$, and $A_{\text{slow}}$ is the limiting change in absorbance at 420 nm. B, semilogarithmic plot of $A_{\text{fast}}/A_{\text{slow}} - A_{\text{t}}$ as a function of time for the initial phase of the reaction shown in A, after subtraction of the contribution of the slowly reacting group.

**Table I**
Rate constants for reaction between adenylate kinase and NBD-chloride

<table>
<thead>
<tr>
<th>Reaction components</th>
<th>$k_{\text{fast}}$</th>
<th>$k_{\text{slow}}$ (Pig)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme</td>
<td>10,500</td>
<td>8,580 205</td>
</tr>
<tr>
<td>Enzyme + AMP</td>
<td>5,430</td>
<td>250</td>
</tr>
<tr>
<td>Enzyme + ATP</td>
<td>3,730</td>
<td>195</td>
</tr>
<tr>
<td>Enzyme + MgATP</td>
<td>3,260</td>
<td>190</td>
</tr>
<tr>
<td>Enzyme + ADP</td>
<td>2,100</td>
<td>2,400 190</td>
</tr>
<tr>
<td>Enzyme + ITP</td>
<td>2,100</td>
<td>2,400 190</td>
</tr>
<tr>
<td>Enzyme + ApA</td>
<td>25</td>
<td>30 120</td>
</tr>
<tr>
<td>Enzyme + MgApA</td>
<td>20</td>
<td>20 150</td>
</tr>
<tr>
<td>Glutathione</td>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

The semilogarithmic plot for the fast reacting group is constructed (Fig. 1B). The rate constant for the fast reaction can be obtained from the slope, as for the slow reaction above. The constants are collected in Table I. The enhanced reactivity of the fast reacting —SH group was lost after denaturation of the enzyme with 0.1% sodium dodecyl sulfate.

**Comparison with Modification by Other Reagents**—Some preliminary studies were performed on the kinetics of reaction of the —SH groups with 5,5'-dithio-bis(2-nitrobenzoic acid). In this case the two —SH groups react at much more similar rates than is the case with NBD-chloride, since the semilogarithmic plot did not show two distinct phases as in Fig. 1A, but rather a gradual continuous curvature. A detailed analysis indicates that the semilogarithmic plot shows two distinct phases only when the ratio of the rate constants of the two groups is greater than about three. Thus it can be concluded that the two —SH groups react at similar (i.e. less than 3-fold different) rates with 5,5'-dithio-bis(2-nitrobenzoic acid).

**Effects of Nucleotides on Rate of Reaction**—The effects of nucleotides on the rate of the enzyme with NBD-chloride are summarized in Table I. In each case the reaction showed a distinct biphasic character, and the kinetic analysis was quite straightforward. AMP, which is known to bind specifically at the "monophosphate" subsite of the enzyme (17), is unique in that it enhances the reactivity of the slowly reacting —SH group. GMP, which is neither a substrate nor an inhibitor of the enzyme (17), has no effect on the rate of reaction of either —SH group. The other nucleotides (except ITP) and MgATP have effects principally on the reaction rate of the fast reacting —SH group. In the presence of ApA, a multisubstrate analogue and a potent inhibitor of adenylate kinase (17, 18), the reactivity of the fast reacting cysteine toward NBD-chloride decreases by a factor of more than 300, whereas the reactivity of the slowly reacting cysteine is not drastically influenced (Table I). Mg(II) and Mn(II) (2 mM) in the absence of nucleotides have no effect on the reaction rate which is not surprising since Mn(II) itself binds only very weakly to the enzyme (17).

**Identification of Sites of Reaction of NBD-chloride**—Attempts to purify peptides which contained NBD-cysteine failed because of the instability of this derivative under the conditions used for separation of the peptides from trypsin digest. The identity of the fast reacting —SH groups was thus inferred indirectly. The fast reacting —SH group of the enzyme solely was modified by reaction with equimolar concentration of NBD-chloride, and the derivative was denatured with 0.1% sodium dodecyl sulfate. An excess of iodo[2-14C]acetate was then added to react with the —SH group which had not been modified by NBD-chloride. After exhaustive dialysis against water to remove excess iodo[2-14C]acetate and the denaturing agent, the sample was dialyzed against buffer containing 1 mM 2-mercaptoethanol (which displaces the NBD group from the enzyme (19)) and then against 10 mM acetic acid. The derivative was lyophilized, carboxymethylated with nonradioactive iodoacetic acid, and digested with trypsin. After digestion the S-carboxymethyl peptides were isolated as previously described (3). The radioactivity is found almost exclusively in peptide T81/92 which contains Cys 187 (Table II). Thus it is clear that Cys-25 is the "fast reacting" —SH group toward NBD-chloride.

Further support for this conclusion was obtained from a study of the enzyme isolated from carp muscle which contains only one —SH group, at the Cys-25 position (11). The reaction rate of the —SH group of this enzyme with NBD-chloride was very similar to that of the fast reacting —SH group of the porcine enzyme, both in the absence and presence of ADP. The second order rate constants for the carp muscle enzyme with NBD-chloride were approximately 10,000 m⁻¹ min⁻¹ and 2,000 m⁻¹.
Transfer of NBD Group on Enzyme—As shown in Fig. 2, the absorption spectrum of the enzyme derivative in which both —SH groups had been modified by NBD-chloride was separated from excess reagent by gel filtration (Sephadex G-25). Modified enzyme (12.7 PM) was incubated in 50 mM K+-Hepes, pH 7.9, at 20°C for the following time periods in minutes: (a) 0; (b) 50; (c) 170; (d) 1900. At the end of the incubation the spectra marked 1, 2, 3, and 4, respectively, were recorded.

Table II

Radioactive assay of peptides after selective carboxymethylation of Cys-187 by iodo[2-'%]acetate

<table>
<thead>
<tr>
<th>Enzyme or derivative</th>
<th>Specific activity</th>
<th>NBD attached</th>
<th>Specific radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate kinase</td>
<td>2100</td>
<td>&lt;25</td>
<td>0.98</td>
</tr>
<tr>
<td>After sequential treatment</td>
<td></td>
<td>&lt;25</td>
<td>0.98</td>
</tr>
<tr>
<td>(1) With NBD-chloride</td>
<td></td>
<td>&lt;25</td>
<td>0.98</td>
</tr>
<tr>
<td>(2) With sodium dodecyl sulfate and iodo[2-'%]acetate</td>
<td></td>
<td>&lt;25</td>
<td>0.98</td>
</tr>
<tr>
<td>(3) Dialysis against 2-mercaptoethanol</td>
<td></td>
<td>580</td>
<td>510</td>
</tr>
<tr>
<td>(4) Carboxymethylation with non-radioactive iodoacetic acid</td>
<td></td>
<td>&lt;25</td>
<td>500</td>
</tr>
<tr>
<td>Peptide T6 (contains carboxymethyl-Cys-25)</td>
<td></td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Peptide T31/32 (contains carboxymethyl-Cys-187)</td>
<td></td>
<td>0</td>
<td>480</td>
</tr>
</tbody>
</table>

Fig. 2. Changes in the absorption spectrum of NBD-labeled adenylate kinase. Enzyme with both —SH groups modified by NBD-chloride was separated from excess reagent by gel filtration (Sephadex G-25). Modified enzyme (12.7 PM) was incubated in 50 mM K+-Hepes, pH 7.9, at 20°C for the following time periods in minutes: (a) 0; (b) 50; (c) 170; (d) 1900. At the end of the incubation the spectra marked 1, 2, 3, and 4, respectively, were recorded.

TABLE III

Nucleotide binding constants and nucleotide quenching of —NH-NBD adenylate kinase fluorescence

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Limiting quenching</th>
<th>Dissociation constant (MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>0.77</td>
<td>370</td>
</tr>
<tr>
<td>ADP</td>
<td>0.81</td>
<td>60</td>
</tr>
<tr>
<td>ATP</td>
<td>0.79</td>
<td>70</td>
</tr>
<tr>
<td>TTP</td>
<td>0.91</td>
<td>149</td>
</tr>
</tbody>
</table>

The concentration of —NH-NBD adenylate kinase was 3 µM (with approximately 0.3 NBD groups per mol of enzyme). Experiments were performed in 50 mM K+-Hepes, pH 7.9, at 20°C with the use of excitation and emission wavelengths of 470 and 530 nm, respectively. The limiting quenching is the ratio of the fluorescence of the fully formed enzyme-nucleotide complex relative to that of the enzyme in the absence of nucleotide. Double reciprocal plots were used to obtain this parameter and the dissociation constant for each nucleotide.
to their binding to the unmodified enzyme (17, 22). Conformational changes in the enzyme may be invoked to explain the effects of the nucleotides on the fluorescence of the modified enzyme since the fluorescence intensity of —NH-NBD adducts is known to be sensitive to the environment (19). ATP, which functions as a substrate for adenylate kinase, quenches the fluorescence of the —NH-NBD enzyme by a smaller amount than do the adenine nucleotides.

**Stoichiometry of NBD Transfer Reaction**—Since the molar absorbancies for both the —S-NBD and —NH-NBD adducts are known (10, 14), it is possible to evaluate the stoichiometry of the transfer process. It can be calculated from the spectra shown in Fig. 2 that approximately 0.65 of the 1.9 NBD groups bound to the enzyme have transferred over the course of 27 hours at pH 7.9. At pH 8.5, e.g. 0.75 NBD groups were transferred within 12 hours.

The number of —S-NBD groups formed after 20 min incubation at room temperature with various molar ratios of NBD-chloride to enzyme and the number of NBD groups subsequently transferred to an amino group after a 24-hour period are listed in Table IV. It will be noted that the amount of —NH-NBD enzyme formed by transfer is high when both cysteine residues (25 and 187) have been modified by NBD-chloride. When Cys-25 is labeled in the carboxyl group of Cys-25 is the sole cysteine residue, only 3% transfer is observed although a not insignificant amount of transfer does occur from the modified fast reacting group (Cys-25) of the porcine enzyme (Table IV). Selective labeling of Cys-187 of the porcine muscle enzyme in the presence of ApA (Table V) led to approximately 50% transfer. The peptides which were derived from adenylate kinase where either Cys-25 or Cys-187 had been labeled by NBD-chloride did not differ from the peptide described above as judged from their composition.

<table>
<thead>
<tr>
<th>Experiment number</th>
<th>Added components</th>
<th>Incubation time</th>
<th>Enzyme activity after incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NBD-Cys residues</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>5 min</td>
<td>mol/mol protein</td>
</tr>
<tr>
<td>2</td>
<td>NBD-chloride</td>
<td>24 hrs</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>ApA</td>
<td>24 hrs</td>
<td>0.61 0.25</td>
</tr>
<tr>
<td>4</td>
<td>ApA + NBD-chloride</td>
<td>36 hrs</td>
<td>0.45 0.50</td>
</tr>
</tbody>
</table>

*In order to shorten the reaction time of the —S-NBD adduct formation, a 20-fold excess of NBD-chloride over enzyme can be used; as soon as absorption measurements indicate that 1.0 —SH group has reacted, the excess of NBD-chloride is removed by dialysis against 50 mM K⁺-Hepes containing 1 mM ApA, pH 7.9, and the transfer is allowed to proceed for the time indicated.

**Identification of ε-NH₂ Acceptor Group of NBD—Adenylate kinase** which was modified with NBD-chloride was left at room temperature for 24 hours to allow transfer of the NBD group to proceed. The derivative was then treated with 1 M 2-mercaptoethanol for 1 hour at 0°C, dialyzed against buffer, and then carboxymethylated with iodoacetate. Digestion with trypsin was performed as described previously (3). Although there was no loss of the NBD group during the digestion in 0.5% ammonium bicarbonate, the isolation of the fluorescent NBD-containing peptide was complicated by two factors: (a) the low solubility of the NBD peptide in most buffer systems used for peptide separation, and (b) the reaction of the NBD group with nucleophilic buffer components as evidenced by the appearance of peptide-free fluorescent bands. Hence use of buffers containing pyridine or primary amines was excluded.

The fluorescent peptide was isolated by a three stage process. The first stage was high voltage paper electrophoresis at pH 2.0 (23), in which the fluorescent band had a mobility (mₑ) of 0.8 relative to serine (24). The second stage utilized descending paper chromatography of the fluorescent band with the use of the solvent system 1-butanol-acetic acid-water (12:3:5, v/v). The center of the resulting broad fluorescent band (RF = 0.5) was subjected to high voltage paper electrophoresis at pH 2.0 in the third stage. The fluorescent band (mₑ = 0.8) was eluted with 50% acetic acid and prepared for amino acid analysis (25). The composition of the peptide was Asp₁, Ser₁, Glu₁, Gly₂, Val₃, Ile₄, Leu₅, Tyr₆, His₇, Arg₉. The composition indicates that the fluorescent band contains peptide T7 (residues 28-31) plus peptide T₈ (residues 32 to 44). The low yield of valine and isoleucine showed that cleavage of the bond between these residues (Ile 28 and Val 29) was not complete in the period of hydrolysis (22 hours). The yield of lysine is low in agreement with the relative stability of the NBD—NH bond against acid hydrolysis.

The final yield of the fluorescent peptide was only 0.1 mol per mol of adenylate kinase. This value is reasonable for paper separation methods but the possibility that residues other than Lys 31 had been labeled cannot be excluded. The peptide was isolated only from NBD-NH-enzyme where both —SH groups had been modified with NBD-chloride. However, the fluorescent peptides which were derived from adenylate kinase where either Cys-25 or Cys-187 had been labeled by NBD-chloride did not differ from the peptide described above as judged from their...
behavior in high voltage paper electrophoresis and paper chromatography.

It was concluded that Lys-31 carried the NBD group and that NBD-Lys-31 was not attacked by trypsin. This conclusion was supported by a comparison of the two-dimensional maps (3) derived from unmodified adenylate kinase and -NH-NBD adenylate kinase. The two maps differed by the weakness of the spots corresponding to the T7 and T8 peptides in the chromatogram of the NBD-NH enzyme. The fluorescence in this latter trypsin peptide map was located in a long strip covering the whole of the peptide fraction which exhibited neutral behavior upon electrophoresis at pH 6.5.

Effect of Modification by NBD-chloride on Enzyme Activity—It has been previously shown that modification of the two —SH groups of the enzyme by either iodoacetate or 1-fluoro-2,4-dinitrobenzene leads to inactivation (6). From these data, it is not possible to distinguish whether inactivation resulted from the modification of one or both —SH groups. Since the rate of reaction with each —SH group was similar, reaction of both —SH groups would be required for complete inactivation in either case. Because of the 40-fold difference in reactivity of the two groups toward NBD-chloride (Table I), an answer may be provided, at least as far as the NBD-chloride modification is concerned. If an equimolar concentration of NBD-chloride is added to the enzyme, the product distribution is 97.5% fast reacting group modified, 2.5% slowly reacting group modified.

The extent of modification of 15 μM enzyme by 15 μM NBD-chloride was followed spectrophotometrically at 420 nm. Aliquots of the reaction mixture at known extents of reaction were withdrawn, quenched by dilution, and assayed for enzymatic activity.

loss in enzymatic activity observed after 12 hours of reaction was due to modification of Cys-25. In that experiment, the absorption spectrum of the product indicated that only cysteine residues had been modified; from the rate constants given in Table I it was estimated that 85% of Cys-187 and 15% of Cys-25 carried an NBD group correlating with the small loss of activity. In the experiment outlined in Table II, carboxymethylation of Cys-187 with no modification at Cys-25 (i.e. after Step 3, 2-mercaptoethanol treatment) causes about 70% loss of enzymatic activity. The large loss is probably due to irreversible structural changes in the enzyme on denaturation.

Since the enzyme remained stable in the presence of ApA (Experiment 4, Table V) the S→N transfer was also studied in the presence of ApA, where the reaction of porcine adenylate kinase with NBD-chloride was mainly restricted to Cys-187. Subsequently a transfer of 0.5 NBD group to a lysine residue was observed; the NBD-NH-enzyme was found to be 98% active when 2 mM 2-mercaptoethanol was present in the assay mixture (Experiment 4, Table V). The NBD-NH adenylate kinase prepared in the absence of ApA (Table V, Experiment 2, 24-hour sample) had only 40% of the original enzymatic activity. However, in control experiments, adenylate kinase carried through all the steps in the absence of NBD-chloride also lost 60% activity (Table V, Experiment 1). The possibility that in the presence of ApA the NBD group was transferred to a lysine residue different from that observed in the absence of ApA was not investigated.

Spin-labeling Studies on Porcine Adenylate Kinase

General Features of EPR Spectrum—The EPR spectrum of a typical spin-labeled preparation of adenylate kinase consists of two types of enzyme-bound spin label of differing mobility (Fig. 4A). The outmost peaks (marked as 1 and 5 in Fig. 4A) arise from a very immobile label, since their separation (60 G) is almost equal to that observed in the powder EPR spectrum of the iodoacetamide spin label (26). The peaks marked 2 and 4 in Fig. 4A arise from a more mobile spin label, and both labels contribute to the amplitude of the center peak marked 3. The modified enzyme did not denature during the course of the EPR experiments since there was no change in the spectrum after 12 hours of incubation at 0°.

Involvement of —SH Groups in Labeling Reaction—The two —SH groups of the enzyme react with 5,5′-dithiobis (2 nitrobenzoic acid) to give an inactive derivative known as adenylate kinase D (8). No reaction of adenylate kinase D occurred with the iodoacetamide spin label under the conditions used for the labeling of the unmodified enzyme (24-hour reaction, 0°). This suggests that the spin labels are attached only to the —SH groups of the enzyme.

Quantitation of Spin-labeling Reaction—The stoichiometry of a spin-labeling reaction is usually difficult to determine, because of the line broadening of the protein-bound label (26). Additional difficulties arise if two (or more) types of bound spin label are present. An approximate procedure for separating the contributions of the two types of label to the EPR spectrum of spin-labeled adenylate kinase is outlined below.

The peak marked 4 in Fig. 4A arises exclusively from the more mobile label attached to the enzyme. The line shape of this peak could be simulated by the corresponding (high field) peak of the free iodoacetamide spin label in glycerol in 62° (Fig. 4B) and the amplitude of the two peaks made to correspond by appropriate adjustments of the instrument gain. Subtraction of the spectrum in Fig. 4B from the total spectrum (Fig. 4A)
should yield the contribution from the immobile label on the enzyme. Since the hyperfine splitting of the spectrum in Fig. 4B is slightly smaller (0.5 G) than that derived from the more mobile spin label in Fig. 4A, the spectrum in Fig. 4B has to be expanded slightly in order to produce the coincidence of the low, center, and high field peaks. The hyperfine splitting of a spin label spectrum is dependent on the polarity of the environment (27), and this may account for the variation in the hyperfine splitting. The spectrum which results from the subtraction procedure shown in Fig. 4C is quite similar to that of the free iodoacetamide spin label in glycerol at 14°C (Fig. 4D). Comparison of the peak amplitudes and instrumental gain settings in the spectra shown in Fig. 4 indicate that there was about 0.8 strongly immobilized spin label and about 0.15 weakly immobilized spin label per mol of enzyme.

The total amount of spin label bound to the enzyme could also be estimated by denaturing the modified enzyme. In 8 M urea, the EPR spectrum of the modified enzyme indicated that only a single type of weakly immobilized label was present (Fig. 4E). This spectrum was similar to that of the free iodoacetamide spin label in glycerol at 55°C. By comparison of the peak amplitudes and instrumental gains, it was estimated that the total amount of bound spin label was about 0.7 mol per mol of enzyme, in reasonable agreement with the amounts of the two types of spin label given above, allowing for the considerable error in estimation of the strongly immobilized spin label. In another preparation of spin-labeled enzyme the concentrations of strongly and weakly immobilized spin labels were estimated by the simulation procedure to be 0.75 and 0.25 spin label per mol of enzyme, respectively. The denaturation method yielded 1.05 spin labels bound per mol of enzyme in this case.

From the data presented it is clear that the reaction of the -SH groups with the spin label is very slow, being incomplete even after 24 hours reaction at 0°C, consistent with the earlier work (6) which showed that the -SH groups of the enzyme were rather unreactive toward iodoacetate. Attempts to increase the amount of labeling by increasing the temperature, the pH of the solution, or the reaction time resulted in increased precipitation of the enzyme. Experiments designed to decide which -SH groups gave rise to each type of bound spin label were not successful.

When the enzyme was treated with an equimolar concentration of NBD-chloride (which modifies Cys-25) and then with the iodoacetamide spin label, the ratio of the amounts of the two types of bound spin label was rather similar to that observed without prior reaction with NBD-chloride. A significant amount of transfer of the NBD label had occurred to the amino group of Lys-31 during the time required for incorporation of spin label as measured by absorption and fluorescence spectra. Such transfer would presumably liberate the -SH group of Cys-25 for subsequent reaction with the iodoacetamide spin label, thus making interpretation ambiguous.

Since the stoichiometry of the total spin label never exceeded 1 mol per mol of protein, it is not possible to distinguish between two possibilities, namely that the two types of spin label spectra, weakly and strongly immobilized, respectively, result (a) from partial labeling of two amino acid residues, or (b) from an equilibrium between two orientations that can be assumed by the spin label on a single amino acid residue, each of the orientations leading to different motional freedom. Such an equilibrium between different orientations of the same spin label has been observed in spin-labeled hemoglobin (28).

**Mobility of Spin Labels Bound to Enzyme**—The two components of spin-labeled adenylate kinase whose spectra are shown in Fig. 4, B and D can be assigned rotational correlation times of 70 ns and 4 ns for the strongly and weakly immobilized spin labels, respectively, by comparison with published spectra (26). From molecular dimensions (4) the tumbling time of the enzyme is calculated to be about 25 ns (at 2°C). Thus within the limits of the error of these quantities, the strongly immobilized spin label would appear to be rigidly held to the enzyme. By contrast the weakly immobilized spin label has considerable freedom of motion, which could arise from rotation about the bonds linking the spin label to the enzyme or from some segmental motions of that part of the enzyme to which the spin label is attached.

**Effect of Nucleotides on Spin Label Spectra**—Addition of nucleotides to the spin-labeled enzyme caused pronounced changes in the EPR spectrum. The effects of AMP, ATP, and MgATP shown in Fig. 5, A, B, and C, respectively, can be interpreted as arising from a pronounced immobilization of the weakly immobilized spin label fraction on binding nucleotide. Small changes in that part of the EPR spectrum arising from the strongly immobilized spin label would be more difficult to detect because of the low amplitudes of the low and high field peaks of this signal.

GTP and MgGTP addition to adenylate kinase caused qualitatively similar changes in the EPR spectrum but smaller than the changes caused by ATP. GMP, which is neither a substrate nor an inhibitor of the enzyme (17), has no effect on the EPR spectrum.

There were no effects of Mg(II) or Mn(II) on the EPR spectra.
of the two -SH groups of the enzyme. Approximately a 40-fold
difference has been found between the rates of reaction of the
two —SH groups with this reagent. Both iodoacetate and 1-fluoro-2,4-dinitrobenzene appear to react at equal rates with the
two groups (6). In reaction with NBD-chloride, the slowly re-
aeting —SH group has a rate constant similar to that of a model
compound, glutathione, whereas the reactivity of the fast reacting
group is comparable to that of the “essential” reactive cyste-
ine residue in apoglyceraldehyde-3-phosphate dehydrogenase
(32). Evidence that the reactivity of the fast reacting —SH

group is a consequence of its microenvironment in the secondary
and tertiary structure of the enzyme is afforded by the loss of its
enhanced reactivity after denaturation. In the denatured en-
zyme, the two —SH groups react at rates comparable to that of

glutathione.

In view of the identification of the fast reacting —SH group as
Cys-25 and the slow one as Cys-187, the properties of the cyste-
ine residues may be examined in terms of the structure deter-
mined by x-ray analysis (4). Since Cys-187 is at the surface of
the molecule, as expected its reactivity toward NBD-chloride is
similar to that of glutathione, and its modification does not lead
to inactivation of the enzyme. On the other hand, Cys-25, the
cysteine which is close to the presumed catalytic center, exhibits
an enhanced reactivity toward NBD-chloride, and its modifica-
tion leads to complete inactivation of the enzyme. However,
the inactivation does not imply that this —SH group is actually
implicated directly in the catalysis, since, as mentioned previously,
derivatives of the enzyme in which both —SH groups have been
modified can be prepared with retention of some activity (3, 6).

Cys-25 could be important in maintaining the active structure of
the enzyme or its modification could interfere with proper bind-
ing of substrate.

Comparative studies on carp muscle adenylate kinase, a dis-
tant relative of the enzyme from pig (11), reveal similar behavior
patterns. The single cysteine residue of carp adenylate kinase
reacts about 20 times faster than model compounds with NBD-
chloride, and 20 times slower with NBD-chloride when ApA is
present (Table I). Like the porcine enzyme, carp adenylate

kine is completely inactivated by a stoichiomet,ric amount of
NBD-chloride; this inhibition can be reversed by thiols. The

cysteine residue in apoglyceraldehyde-3-phosphate dehydrogenase
(32). Evidence that the reactivity of the fast reacting —SH
group is a consequence of its microenvironment in the secondary
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tion leads to complete inactivation of the enzyme. However,
the inactivation does not imply that this —SH group is actually


**DISCUSSION**

NBD-chloride is so far unique in differentiating the reactivities
of the two —SH groups of the enzyme. Approximately a 40-fold

\[ \text{value of } \tau_r \text{ is assumed to be the same as for creatine kinase} \]

\[ \text{(30), since the EPR spectra of the E-MnATP for adenylate kinase} \]

\[ \text{(17) and for creatine kinase (31) are almost the same.} \]

Fig. 5. A, effect of AMP (5.5 mM) on the EPR spectrum of spin-
labeled adenylate kinase. The spectrum was recorded under
the same conditions as described for Fig. 4A, but the addition of
nucleotide has caused a 10% dilution of the spin-labeled enzyme.
B, effect of ATP (4 mM) on the EPR spectrum of spin-labeled
adenylate kinase. The spectrum was recorded under the same
conditions as described for Fig. 4A, with a 10% dilution of the
spin-labeled enzyme. C, effect of MgATP on the EPR spectrum
of spin-labeled adenylate kinase. The concentrations of Mg(II)
and ATP were 7.5 mM and 3.4 mM, respectively. Conditions as
for Fig. 4A, but with an over-all 30% dilution of the spin-labeled
enzyme.

Fig. 6. Comparison of MnATP (---) and MgATP (--) on
the EPR spectrum of spin-labeled adenylate kinase. The
concentrations of Mn(II) and ATP were 2.3 mM and 3.4 mM,
respectively, and the MgATP spectrum is the same as 5C. Conditions
as for Fig. 4A with an over-all 30% dilution of the spin-labeled
enzyme.

**Fig. 5.** A, effect of AMP (5.5 mM) on the EPR spectrum of spin-
labeled adenylate kinase. The spectrum was recorded under
the same conditions as described for Fig. 4A, but the addition of
nucleotide has caused a 10% dilution of the spin-labeled enzyme.

B, effect of ATP (4 mM) on the EPR spectrum of spin-labeled
adenylate kinase. The spectrum was recorded under the same
conditions as described for Fig. 4A, with a 10% dilution of the
spin-labeled enzyme. C, effect of MgATP on the EPR spectrum
of spin-labeled adenylate kinase. The concentrations of Mg(II)
and ATP were 7.5 mM and 3.4 mM, respectively. Conditions as
for Fig. 4A, but with an over-all 30% dilution of the spin-labeled
enzyme.

of spin-labeled enzyme in the absence of nucleotide. The effect
of MnATP on the EPR spectrum of spin-labeled enzyme was also
studied and compared with the effect of MgATP (Fig. 6). It was
found that there was a small (approximately 20%) diminution in
the amplitude of the center peak in the presence of MnATP
compared with MgATP which appears to be associated primarily
with the highly immobilized spectrum. The distance between
Mn(II) and the unpaired electron of the strongly immobilized
spin label can be estimated on the basis of the theory (29) for the
effect of a paramagnetic ion on the EPR spectrum of a spin label
when both are rigidly attached to a macromolecule. The con-
tribution of the strongly immobilized spin label and thus the
lower limit for the decrease in amplitude of this component caused
by MnATP compared with MgATP is 20%. Using a value of
\( \tau_r \) (the electron spin relaxation time) of \( \approx 1 \times 10^{-9} \) s for the Mn-
(II) ion in the enzyme-MnATP complex it is possible to place
an upper limit of 18 A on the distance between the Mn(II) ion
and strongly immobilized spin label in the complex (30). This
estimate is subject to considerable error chiefly due to the diffi-
culty in estimating what fraction of the decreased amplitude of
the center peak on addition of MnATP should be attributed to the
strongly immobilized component and some uncertainty in the
value of \( \tau_r \). The distance estimated is consistent with the
tentative conclusion (4, 19) that one of the —SH groups of the
enzyme may be close to the binding site for the enzyme sub-
strates.

\[ \text{The value of } \tau_r \text{ is assumed to be the same as for creatine kinase} \]

\[ \text{(30), since the EPR spectra of the E-MnATP for adenylate kinase} \]

\[ \text{(17) and for creatine kinase (31) are almost the same.} \]
on the reactivity of the cysteine residues toward NBD-chloride and on the EPR spectrum of the spin-labeled enzyme. Changes in reactivity of amino acid residues cannot be interpreted unambiguously in terms of conformational changes in the absence of detailed structural information. It is possible that the nucleotides could be exerting a direct "masking" effect on the —SH groups which would hinder the approach of reagent rather than inducing conformational changes in the enzyme. Clearly the explanation is more plausible for the effect of AMP where an enhancement of reactivity of the slowly reacting —SH group is observed. The changes in EPR spectrum of the enzyme bound spin label upon addition of nucleotides provide more direct evidence for conformational changes in the enzyme in the region of the spin label. Cogent evidence for more widespread substrate-induced conformational changes is afforded by proton-NMR studies of the enzyme.

The availability of the detailed structure of crystalline adenylate kinase permits us to examine the phenomenon of NBD transfer from an —SH to an —NH₂ in structural terms. The results reported here indicate that transfer proceeds predominantly from Cys-187. This conclusion is in agreement with the structure of crystalline adenylate kinase. The sulfur atom of Cys-187 is separated from the e-N atom of Lys-31 only by solvent, the distance between the two atoms being approximately 4 A (4). However, it should be noted (Table IV) that some transfer of the NBD group occurred when only the fast reacting —SH group (Cys-95) of the enzyme had been modified. A direct transfer is difficult to reconcile with the crystal structure data since the sulfur atom of Cys-25 and nitrogen atom of Lys-31 are some 15 A apart. If it is assumed that the transfer of NBD from Cys-25 is indeed to Lys-31 there are two possible explanations to account for the transfer in the singly modified enzyme. (a) Reaction of NBD-chloride with Cys-25 induces a conformational change in the enzyme which brings Lys-31 into closer proximity. (b) The NBD group might be transferred from Cys-25 to Cys-187 (via an inter- or intramolecular process) and thence to Lys-31. In the crystal structure the sulfur atom of Cys-187 is 13 A from the sulfur atom of Cys-25.

The first possibility seems unlikely in view of the fact that in carp muscle adenylate kinase which lacks Cys-187 but retains both Cys-25 and probably also Lys-31 (11) there appears to be very little transfer of the NBD under these conditions (Table IV). The second possibility could readily account for the apparent anomaly since in an intermolecular process Cys-187, like other thiols (18), could react with NBD-Cys-25. An intramolecular transfer of the NBD group, however, cannot be excluded since evidence from a number of studies, such as oxygen quenching of fluorescence (35), fluorescence polarization (34), high resolution nuclear magnetic resonance (35), and spin labeling (36) indicates that proteins in solution can possess considerable flexibility.

At this point it might be of value to compare the results which were obtained from studies on crystalline adenylate kinase at pH 7.7 in the presence of 3 m (NH₄)₂SO₄ by x-ray analysis (4) and the results reported here. The milieu used in our experiments (10 μM protein, 90 mM buffer of pH 7.9 at 20-30°) might be regarded as approaching physiological conditions. As predicted by the x-ray analysis (4) our data suggest that Cys-25 is the cysteine which is close to the presumed catalytic center of the enzyme.

Both the x-ray data and the S → N transfer of the NBD group suggest a close proximity of Cys-187 and Lys-31. Therefore, it can be concluded that the geometry of this section of the protein molecule is the same for the enzyme in solution and the enzyme in the crystal.

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