Reaction of Low Molecular Weight Aminothiols with o-Phthalaldehyde

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o-Phthalaldehyde has been recently shown to be a useful reagent for chemical modification of cyclic nucleotide dependent protein kinases, hexokinase, and fructose-1,6-bisphosphatase. It reacts covalently with closely spaced (~3 Å) sulphydryl and ε-amino functions of cysteine and lysine residues, respectively, of these enzymes to yield fluorescent isoindole derivatives. We have found the reagent to be equally useful to investigate the degree of reactivity of sulphydryl and amino functions in substances that do not possess enzymatic activity, e.g., glutathione, homocysteine, and cysteine. The kinetics of the reaction of nonenzymatic aminothiols with o-phthalaldehyde can be followed rapidly and conveniently by continuously monitoring the increase in relative fluorescence of the isoindole derivatives. The fluorescence emission maxima of the o-phthalaldehyde adducts can be used to compute molar transition energies that provide qualitative but useful information concerning the degree of polarity of microenvironment of the sulphydryl and amino functions participating in isoindole formation. The kinetic and spectral data obtained from the reaction between o-phthalaldehyde and nonenzymatic low molecular weight aminothiols may be helpful in comparing the reactivities of the sulphydryl and amino functions in enzymes.

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KEY WORDS: o-phthalaldehyde; aminothiols; kinetics; fluorescence; molar transition energy.

Previous reports from our laboratory described the inactivation of the catalytic subunit of cAMP-dependent protein kinase (1), cGMP-dependent protein kinase (2), hexokinase (3,4), and fructose-1,6-bisphosphatase (5) by o-phthalaldehyde. In all these cases, two important findings are that (a) o-phthalaldehyde reacts with ε-NH₂ and SH functions of those lysines and cysteines, respectively, whose spatial proximity is similar to that between the two vicinal aldehydic functions (~3 Å) in the modifying reagent (Fig. 1), and (b) the cysteine and lysine residues reacting with o-phthalaldehyde are located in the hydrophobic environment of these enzymes.

Although the reaction of o-phthalaldehyde with thiols in the presence of primary amines to yield fluorescent isoindole derivatives (6–8) has been known for some time and forms the basis for detection of amino acids by post-column derivatization in sequence analysis (for review, see Ref. (9)), its potential use in the chemical modification of proteins has been due largely to the efforts of our laboratory (1–5). Aminothiols such as glutathione and N-acetylcysteine have been used as models for comparing the reactivity of the SH groups in enzymes (10). However, because of their nonenzymatic character, it is difficult to investigate the rates of their reactions with modifying reagents. Therefore, we investigated the reaction of the above nonenzymatic low molecular weight aminothiols with o-phthalaldehyde. The spectrofluorometric
method described in this paper is simple and rapid and may be used with equal ease to investigate the reaction kinetics of \( \alpha \)-phthalaldehyde with enzymatic and nonenzymatic substances under similar conditions.

MATERIALS AND METHODS

Glutathione, glutathione disulfide, \( S \)-methyl glutathione, cysteine, cystine, homocysteine, homocysteine, \( S \)-methylcysteine, methionine, NADP\(^+\), glucose, \( \alpha \)-phthalaldehyde, glucose-6-phosphate dehydrogenase, hexokinase (yeast), and creatine kinase (rabbit muscle) were obtained from Sigma. Commercial samples of yeast hexokinase used in this work were judged homogeneous by electrophoresis in the presence and absence of sodium dodecyl sulfate. \( \alpha \)-Phthalaldehyde solutions were prepared in methanol prior to use and the concentration determined spectrophotometrically as described in (11). The final concentration of methanol in the incubation mixtures was 1\% (v/v). Hexokinase concentration was determined by using a specific absorption of 0.947 cm\(^2\) mg\(^{-1}\) (12).

Hexokinase activity was measured spectrophotometrically by coupling with glucose-6-phosphate dehydrogenase and following reduction of NADP\(^+\) at 340 nm (13). Reaction of hexokinase, creatine kinase, glutathione, homocysteine, and cysteine with \( \alpha \)-phthalaldehyde was monitored spectrophotometrically essentially as described by Rossi et al. (14). An appropriate enzymatic or nonenzymatic substance was incubated with \( \alpha \)-phthalaldehyde and the increase in relative fluorescence was measured at various time intervals. Percentage of activity or reactivity of any enzymatic or nonenzymatic substance, respectively, remaining following incubation with \( \alpha \)-phthalaldehyde is given by \( (F_\infty - F_t)/(F_\infty - F_0) \), where \( F_0 \), \( F_r \), and \( F_\infty \) represent relative fluorescence at zero time, at any given time \( t \), and the maximum fluorescence.

Relative fluorescence values for hexokinase, creatine kinase, glutathione, homocysteine, and cysteine were recorded at 405, 400, 410, 455, and 440 nm, respectively. These wavelengths represent fluorescence emission maxima of the \( \alpha \)-phthalaldehyde adduct of these substances following excitation at 338 nm characteristic of an isoindole ring (1,6–8).

Fluorescence measurements were made with a SLM 4800 spectrofluorometer interfaced with a Hewlett-Packard Model HP 9825A desk top calculator and Hewlett-Packard Model 7225B plotter. The SLM PR-8015 spectrum processor program was used for recording both fluorescence excitation and emission spectra. Excitation and emission slit widths were 4 nm and all operations were performed at 25°C. Spectra were recorded in a microcuvette of 3-mm path length.

RESULTS AND DISCUSSION

\( \alpha \)-Phthalaldehyde reacts with free thiols in the presence of primary amines to yield isoindoles with characteristic fluorescence excitation (\( \lambda_{ex} \), 338–343 nm) and emission (\( \lambda_{em} \), 397–434 nm) maxima (6–8). The stability of the isoindole derivatives varies with substituents on the isoindole ring (7,15). However, due to the constraints imposed by the backbone in the tertiary structure of proteins, the SH and \( \text{NH}_2 \) groups must be oriented in a way to provide the necessary spatial requirement (\( \sim 3 \) Å) to react with the vincinal dialdehyde functions in \( \alpha \)-phthalaldehyde (1) (Fig. 1b). We demonstrated that mobility of

![Fig. 1. Schematic representation of the reaction of \( \alpha \)-phthalaldehyde with (a) glutathione and (b) with an enzyme.](image-url)
these residues allows their coming together up to this distance. We also demonstrated that, in proteins, only the \(\epsilon\)-NH\(_2\) group donated by lysine residues participates in isindole formation during reaction with \(\alpha\)-phthalaldehyde (1). Whereas aminothiols, such as glutathione, cysteine, and homocysteine, form isindole derivatives with \(\alpha\)-phthalaldehyde in the absence of an added thiol, cysteamine and glycine require the addition of an external thiol, e.g., \(\beta\)-mercaptoethanol (Table 1). Quantum yield of the isindoles derived from cysteine and homocysteine was found to be considerably less than the yield derived from glutathione. These results are in complete accord with those of Cohn and Lyle (16). One of the reasons that cysteine and homocysteine, but not cysteamine, form isindoles with \(\alpha\)-phthalaldehyde may be attributed to the presence of a chiral center in the former compounds. Thus, a certain population of conformers in cysteine and homocysteine may have the right spatial orientation of SH and NH\(_2\) functions necessary to react with \(\alpha\)-phthalaldehyde. The ability of glutathione, cysteine, and homocysteine to react with \(\alpha\)-phthalaldehyde has been previously demonstrated by us (1). The fact that a free sulphydryl function in glutathione, cysteine, and homocysteine forms isindoles with \(\alpha\)-phthalaldehyde is confirmed by the fact that glutathione disulfide, \(S\)-methyl glutathione, methionine, cystine, \(S\)-methylcysteine and homocysteine did not yield isindole derivatives when examined by fluorescence spectroscopy. The fluorescence emission (\(\lambda_{em} = 410\) nm) and excitation spectra (\(\lambda_{em} = 355\) nm) of the glutathione–\(\alpha\)-phthalaldehyde ad-

### TABLE 1

**Fluorescence Emission Maxima and Molar Transition Energies of the Isindole Derivatives Formed in Reaction of the Following with \(\alpha\)-Phthalaldehyde**

<table>
<thead>
<tr>
<th>Reactant</th>
<th>Solvent</th>
<th>Fluorescence emission ((\lambda_{em}, \text{nm}))</th>
<th>Molar transition energy (kJ/mol)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EA(^b)</td>
<td>Water</td>
<td></td>
<td>267</td>
<td>Palczewski et al. (11)</td>
</tr>
<tr>
<td>EA(^b)</td>
<td>Methanol</td>
<td></td>
<td>233</td>
<td>Palczewski et al. (11)</td>
</tr>
<tr>
<td>EA(^b)</td>
<td>Hexane</td>
<td></td>
<td>127</td>
<td>Palczewski et al. (11)</td>
</tr>
<tr>
<td>cGMP-dependent</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>405</td>
<td>121</td>
<td>Puri et al. (2)</td>
</tr>
<tr>
<td>protein kinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>403</td>
<td>116</td>
<td>This paper</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>400</td>
<td>113</td>
<td>This paper</td>
</tr>
<tr>
<td>Glutathione</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>410</td>
<td>136</td>
<td>This paper</td>
</tr>
<tr>
<td>Cysteine</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>440</td>
<td>226</td>
<td>This paper</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>455</td>
<td>274</td>
<td>This paper</td>
</tr>
<tr>
<td>(\beta)-Mercaptoethanol + glycine</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>440</td>
<td>226</td>
<td>This paper</td>
</tr>
<tr>
<td>(\beta)-Mercaptoethanol + cysteamine</td>
<td>HEPES–NaOH, pH 7.3</td>
<td>445</td>
<td>241</td>
<td>This paper</td>
</tr>
</tbody>
</table>

\(^a\) Molar transition energies were calculated by using the equation \(E_T = 2.985 \lambda_{em} - 1087.28\) as previously described by Puri et al. (1).

\(^b\) EA, 1-[(\(\beta\)-Hydroxyethyl)thio]-2-(\(\beta\)-hydroxyethyl)isoindole.
duct, typical of isoindole derivatives (1,2,5), are shown in Figs. 2A and 2B, respectively, and are very similar to spectra previously reported (16). The fluorescence emission maximum of the glutathione-o-phthalaldehyde adduct, interestingly, resembles more closely those of the adducts of cGMP-dependent protein kinase (2), hexokinase (4), and creatine kinase than those of cysteine, homocysteine, cysteamine + β-mercaptoethanol, and glycine + β-mercaptoethanol (1) (Table 1). This implies that the structure of glutathione may present its SH and NH₂ functions environment similar to those present in the highly organized and complex structures of the above enzymes (see below).

The linear free energy relationship (17) between the molar transition energy \( E_T \) and fluorescence emission maximum developed by Dimroth et al. (18) has been used by us (1–5) and others (11) to probe the degree of polarity of the microenvironment of the cysteine and lysine residues participating in isoindole formation with various enzymes. The results of such an investigation of the o-phthalaldehyde adduct of various substances are summarized in Table 1. The \( E_T \) values of the o-phthalaldehyde adducts of glutathione, creatine kinase, hexokinase, and cGMP-dependent protein kinase (1) are similar to that of a model synthetic isoindole, 1-[(β-hydroxyethyl)thio]-2-(β-hydroxyethyl)isoindole (EA) in hexane. It has been previously demonstrated that SH and NH₂ functions participating in isoindole formation in hexokinase (4,5), the catalytic subunit of cAMP-dependent protein kinase (1), and cGMP-dependent protein kinase (2) are located at or near the active site of these enzymes. Fluorescence polarization studies from our laboratory have shown that the active sites of cAMP-dependent protein kinase (19), cGMP-dependent protein kinase (20), and hexokinase (21) are located in a hydrophobic environment. That the \( E_T \) value of the glutathione adduct resembles those of the above enzymes may be explained in two ways. First, it is possible that folding of the glutathione molecule, during isoindole formation in reaction with o-phthalaldehyde, could provide insulation of the isoindole ring by the hydrocarbon chain of glutamic acid residue of the tripeptide, thus providing a hydrophobic environment to the isoindole ring. It has been previously demonstrated that bulky nonpolar t-butyl groups around isoindole not only cause a blue shift in the fluorescence emission maximum (presumably by creating a nonpolar environment) but also insulate the isoindole ring from interaction with the solvent (7,15). Alternatively, the properties of the glutathione–o-phthalaldehyde adduct are the result of restricted solvent relaxation during the lifetime of the first excited singlet state (blue shift in the emission spectrum) (22). However, in the absence of data concerning

![Figure 2](image-url)
the difference between the dipole moments of the excited and ground states of the glutathione-o-phthalaldehyde adduct during fluorescence emission, the solvent relaxation effect contributing to the blue shift of the fluorescence emission maximum of the glutathione adduct cannot be ruled out as a distinct possibility. But, certainly this argument is less attractive in the case of cyclic nucleotide-dependent protein kinases and hexokinase (19-21), where the hydrophobic environment of the domain containing SH and NH₂ groups participating in isoindole formation has been amply demonstrated (19,21). Nevertheless, the $E_1$ values for the o-phthalaldehyde adduct with cysteine, homocysteine, β-mercaptoethanol + glycine, and β-mercaptoethanol + cysteamine are similar to that of EA in methanol or water.

Glutathione and N-acetylcysteine have been used as models for the reactivity of the SH groups in yeast hexokinase (10). However, the kinetic experiments used to study the rates of reaction of glutathione and N-acetylcysteine with modifying reagents are cumbersome and time-consuming (10). We used the spectrofluorometric method to study the rates of reaction of glutathione, homocysteine, and cysteine with o-phthalaldehyde. In order to do so, we first investigated the reaction kinetics of the inactivation of hexokinase by o-phthalaldehyde using activity and relative fluorescence measurements. In the spectrofluorometric assay, the increase in relative fluorescence is a measure of the progress of isoindole formation between hexokinase and o-phthalaldehyde. The fraction $(F_\infty - F_i)/(F_\infty - F_0)$ is a measure of the hexokinase activity remaining at any given time, $t$ (see Materials and Methods). A plot of the logarithm of percentage of activity remaining or percentage of increase in relative fluorescence against time yields the pseudo-first-order rate constant for the inactivation of hexokinase. Such a plot is shown in Fig. 3. The pseudo-first-order reaction rates for the inactivation of hexokinase by o-phthalaldehyde by activity and relative fluorescence measurement are identical at that concentration of o-phthalaldehyde. The second-order rate constant for the inactivation of hexokinase is obtained from the slope of the plot of the pseudo-first-order rate constants versus concentration of o-phthalaldehyde (4) (also see Fig. 4). The second-order rate constants for the inactivation of hexokinase by o-phthalaldehyde as determined by activity measurement (3,4) and the relative fluorescence method (this paper) were identical, i.e., 45 M⁻¹ s⁻¹. The relative fluorescence method is
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Fig. 4. Time course of reaction between glutathione (reduced) and o-phthalaldehyde. Glutathione (150 ng/100 µl) was incubated with o-phthalaldehyde as described in Fig. 3. The progress of the reaction was monitored spectrofluorometrically (λem = 410 nm) as described in Fig. 3. o-Phthalaldehyde concentrations were (Δ) 0, (■) 0.8, (□) 1.6, (●) 2.4, and (○) 3.2 mM. The inset shows a plot of pseudo-first-order rate constants vs concentration of o-phthalaldehyde. The second-order rate constant was obtained from the slope of the secondary plot.

Continuous and rapid. It does not require quenching of the incubation mixture and measurement of enzyme activity at each time point. The design of this experiment and results obtained emphasize the fact that, indeed, inactivation of hexokinase by o-phthalaldehyde is a direct consequence of an isoindole derivative formation. The second-order rate constant determined spectrofluorometrically is the true second-order rate constant of inactivation of hexokinase by o-phthalaldehyde and hence demonstrates the validity of this approach. Similar results have been obtained during investigations involving the inactivation of the catalytic subunit of cAMP-dependent protein kinase (1) and aldolase (11). It is, therefore, reasonable to extend the spectrofluorometric method to determine the rate of isoindole derivative formation between low molecular weight aminothiols and o-phthalaldehyde. The results of reaction kinetics of the isoindole formation between glutathione and o-phthalaldehyde are shown in Fig. 4. The results of other aminothiols are presented in Table 2. The second-order rate constant for the formation of the glutathione-o-phthalaldehyde adduct was found to be 25 M⁻¹ s⁻¹ and compares with those of hexokinase (3,4), cGMP-dependent protein kinase (2), and creatine kinase. Similar conclusions have been reached by other investigators (10) who compared the reactivity of the SH function of glutathione with that of hexokinase and creatine kinase. The second-order rate constants for the reaction of o-phthalaldehyde with homocysteine and cysteine are much higher. The results obtained from kinetic experiments can be explained in terms of the ET values. The SH and NH₂ groups in glutathione, hexokinase, cGMP-dependent protein kinase, and creatine kinase, because of their hydrophobic environment, are not readily accessible to the

Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K (M⁻¹ s⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>cGMP-dependent protein kinase</td>
<td>25°</td>
<td>Puri et al. (2)</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>45h°</td>
<td>Puri et al. (4)</td>
</tr>
<tr>
<td>Creatine kinase</td>
<td>35°</td>
<td>This paper</td>
</tr>
<tr>
<td>Glutathione</td>
<td>25°</td>
<td>This paper</td>
</tr>
<tr>
<td>Cysteine</td>
<td>220°</td>
<td>This paper</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>236°</td>
<td>This paper</td>
</tr>
</tbody>
</table>

° Solutions containing the specified components (3-200 µM), 100 mM Hepes-NaOH, pH 7.3, 0.1 mM EDTA, 1% methanol, and o-phthalaldehyde were incubated at 25°C.

° Kinetics experiments were performed using activity measurements.

° Kinetics experiments were performed using fluorescence measurements (see Figs. 2 and 3).
modifying reagent and thus exhibit lower values for their second-order rate constant. Nevertheless, it is clear that a small tripeptide-like glutathione serves as a good model for comparing the reactivity of the sulfhydryl groups of hexokinase or even cGMP-dependent protein kinase.

In summary, we have shown that (a) o-phthalaldehyde can be used to study the reactivity of low molecular weight aminothiols, (b) the spectrofluorometric method for investigating the kinetics of the reaction between low molecular weight aminothiols and o-phthalaldehyde is simple and rapid, and (c) the molar transition energies ($E_\text{r}$) obtained from the fluorescence emission spectra of o-phthalaldehyde adducts provide qualitative but useful information about the degree of polarity of the microenvironment of functional groups in aminothiols reacting with o-phthalaldehyde.

ACKNOWLEDGMENTS

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