Fluorescence Energy Transfer Experiments with *Escherichia coli* Carbamoyl-phosphate Synthetase†

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ABSTRACT: Fluorescence energy transfer experiments were used to measure distances between three fluorescently labeled sulfhydryl sites on *Escherichia coli* carbamoyl-phosphate synthetase, an unsymmetrical dimer. When five different combinations of fluorescent donor–acceptor pairs are used, the distance between site 1, located on the large subunit, and site 2, located on the small subunit, is in the range of 27–33 Å. Similarly, the distance between site 1 and site 3 (large subunit) was ~27 Å and between site 2 and site 3 was ~21 Å. A similar approach was employed to determine distances between each sulfhydryl group and the ATP site(s), and in all cases no fluorescence quenching was observed using Cr^{3+}ATP or $Co(NH_3)_4$ATP as substrate analogues. A lower limit could be calculated from these data, resulting in a distance of ≥21 Å from each sulfhydryl site to the ATP site. Additional experiments were performed to evaluate if the substrates ATP, HCO_3^-, or glutamine or the allosteric modifiers ornithine, IMP, and UMP altered the distance relationships among the sulfhydryl sites. IMP and UMP produced a slight decrease in fluorescence between sites while glutamine and ATP produced a slight increase in fluorescence.

Previous work on carbamoyl-phosphate synthetase from *Escherichia coli* (Matthews & Anderson, 1972; Foley et al., 1971) has established that three sulfhydryl groups can be modified by *N*-ethylmaleimide. The enzyme has a molecular weight of 180000 and is composed of two nonidentical subunits (Matthews & Anderson, 1972; Trotta et al., 1971), one of molecular weight 48000 and the other 130000.

Carbamoyl-phosphate synthetase catalyzes the reaction

$$2MgATP + HCO_3^- + \text{L-glutamine} \rightarrow 2MgADP + P_i + \text{L-glutamate} + \text{carbamoyl phosphate} \quad (1)$$

The smaller subunit contains the binding site for glutamine while the larger subunit contains the binding sites for the rest of the substrates and allosteric modifiers (Trotta et al., 1971).

EPR[1] data from this laboratory (Raushel et al., 1979) demonstrated that the three reactive sulfhydryl groups can be derivatized by maleimide spin-labels. One sulfhydryl is on the small subunit while the other two are on the large subunit. The spin-label environment of these three enzymes was sensitive to some substrates but insensitive to allosteric modifiers. The spatial relationship among the three reactive sulfhydryl groups could not be determined from these studies.

In this paper we report data on fluorescent maleimide compounds that were used to derivatize each of the three sulfhydryl groups. From fluorescence energy transfer experiments, we were able to establish the spatial relationships among the three sites and how they respond to binding substrates and allosteric modifiers. These data along with those in the preceding paper (Raushel et al., 1983) represent the beginnings of a topographical map of the active site and allosteric sites of this important regulatory enzyme.

Materials and Methods

Carbamoyl-phosphate synthetase was isolated from *E. coli* according to the method of Matthews & Anderson (1972). The fluorescent NEM probes were obtained from the following companies: NPM was purchased from Fluka Chemical Corp.; NBPM was from Eastman Kodak Co.; CPM was from Molecular Probes; DDPM and QS were from Aldrich Chemical Co.; and NBD-Cl was from Sigma Chemical Co. DTNB was also obtained from Sigma. β,γ-CrATP was prepared according to the procedure of DePamphilis & Cleland (1973) and Dunaway-Mariano (1978). β,γ-$Co(NH_3)_4$ATP was synthesized by the method of Cornelius et al. (1977). All other biochemicals were obtained from Sigma.

Enzyme Assays. Carbamoyl-phosphate synthetase activity was measured spectrophotometrically by using a pyruvate kinase–lactate dehydrogenase coupling system. A Hitachi Model 100-80 computerized UV/vis spectrometer equipped with a Haake FS water bath was used to follow the reaction at 340 nm. For specific activity measurements, each 1.0-mL cuvette contained 50 mM Hepes, pH 8.2, 33 μg each of salt-free lactate dehydrogenase and pyruvate kinase, 0.2 mM NADH, 10 mM KCl, 10 mM ornithine, 15 mM excess $MgCl_2$, and various amounts of different substrates and inhibitors. Assays were conducted at 37 °C and carbamoyl-phosphate synthetase (5–10 μg) was added last to initiate the reaction.

Preparation of Labeled Carbamoyl-phosphate Synthetase. For the preparation of *N*-ethylmaleimide derivatives of three different sulfhydryl groups on the enzyme, the procedures of Foley et al. (1971) and Matthews & Anderson (1972) were followed with slight modification. In accordance with the nomenclature of Foley et al. (1971), these derivatives have been named CPS–$label_1$, CPS–$label_2$, and CPS–$label_3$.

CPS–$Label_1$. In a volume of 0.5 mL, carbamoyl-phosphate synthetase (5.1 mg/mL) was incubated with 130 mM potassium phosphate buffer (pH 7.5), 13 mM L-ornithine, and 0.35 mM maleimide probe at 17 °C for 2 h. The unreacted label was removed by elution through a small Sephadex G-

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CPS, carbamoyl-phosphate synthetase; NEM, *N*-ethylmaleimide; NPM, *N*-(3-pyrenyl)maleimide; NBPM, *N*-[*p*-(2-benzoxazolyl)phenyl]maleimide; CPM, 3-(4-maleimidylphenyl)-7-(diethylamino)-4-methylcoumarin; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); QS, quinine sulfate; DTE, dithioerythritol; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EPR, electron paramagnetic resonance.

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25-80 column (0.9 cm i.d. × 13 cm). Because of the different enzyme environments of the three sulfhydryls the extinction coefficients of model NEM derivatives could not be used to assess the extent of covalent labeling. Instead, the stoichiometry of labeling was determined by titration with DTNB according to the procedure of Foley et al. The protein concentrations were determined by the method of Lowry et al. (1951).

CPS–Label$_2$. For derivatization of the "second" sulfhydryl group with either DTNB or one of the other labels, the "first" sulfhydryl (CPS–label$_1$) was initially labeled following the procedure of Matthews & Anderson (1972). In a volume of 0.5 mL, carbamoyl-phosphate synthetase (10.2 mg/mL) was incubated with 130 mM potassium phosphate buffer (pH 7.5), 10 mM L-ornithine, and 0.70 mM DTNB or one of the other probes for 1 h at 17 °C. The unreacted label was removed by elution through a Sephadex column. The protein was concentrated by ultrafiltration and the extent of labeling determined by DTNB titration. Then, in a volume of 1.0 mL, 4.6 mg of the derivatized enzyme was incubated with 10 mM ATP, 10 mM $MgCl_2$, 10 mM HCO_3^-, and 0.41 mM of the next label for 2 h at 17 °C. Elution through a Sephadex column followed and the extent of labeling was determined as described above.

CPS–Label$_3$. To derivatize the "third" sulfhydryl, the first two SH groups were reacted by the following procedure. In a volume of 0.5 mL, carbamoyl-phosphate synthetase (10.2 mg/mL) was incubated with 130 mM potassium phosphate buffer, pH 7.5, 13 mM L-ornithine, and 0.7 mM DTNB or maleimide probe at 17 °C for 2 h. The unreacted label was removed as before and the protein was concentrated by ultrafiltration. In a volume of 1.0 mL, 4.5 mg of this modified enzyme derivative was incubated with 10 mM ATP, 10 mM $MgCl_2$, 10 mM HCO_3^-, and 0.41 mM of the next label for 2 h at 17 °C followed by elution through a Sephadex column and concentration. Next, in a volume of 1.0 mL, 4.1 mg of the doubly derivatized enzyme was incubated with 130 mM potassium phosphate buffer, pH 7.5, and 0.75 mM of the third probe for 2 h at 17 °C. Elution and concentration followed as before. When the first two SH groups were labeled with identical probes, the procedure was modified slightly. Carbamoyl-phosphate synthetase (5.1 mg/mL) was placed in a volume of 1.0 mL that included 130 mM potassium phosphate, pH 7.5, 10 mM ornithine, 10 mM ATP, 10 mM HCO_3^-, 10 mM $MgCl_2$, and 0.75 mM of the appropriate probe. Incubation proceeded for 1 h at 17 °C and was terminated by elution through a Sephadex column and ultrafiltration. This procedure was followed by incubation of 4.1 mg of this modified enzyme derivative in a volume of 1.0 mL containing 130 mM potassium phosphate buffer, pH 7.5, and 0.75 mM probe for 2 h at 17 °C. Elution and concentration followed. When labeling with DTNB, incubation times were 25 min instead of the 1- or 2-h period. To remove DTNB from an enzyme labeled with this compound, nearly stoichiometric amounts of DTE were added to the derivatized enzyme. After this treatment, the enzyme was concentrated as before. The extent of regeneration of free SH from DTNB-labeled enzyme was determined by titration of an aliquot of enzyme with DTNB.

Spectroscopic Measurements. UV–vis spectra were determined with a Hitachi 100-80 spectrophotometer equipped with a thermostated cell holder. Steady-state fluorescence and polarization measurements were made with a Perkin-Elmer MPF-44B fluorescence spectrometer equipped with polarization accessories and thermostated to 25 °C. All samples were passed through a 0.8-μM Millipore-brand filter before spectra were measured. The method of Parker & Rees (1966) was used to determine the quantum yield of samples in 50 mM Hepes at pH 7.5, 25 °C. Equation 2 gives the ratio of

$$Q_2/Q_1 = (F_2/F_1)(A_1/A_2) \quad (2)$$

quantum yields, Q_i's, as a function of the area of the emission spectrum, F_i, and the absorbance at the exciting wavelength, A_i, for two different fluorescent compounds. Quinine sulfate in 0.1 N H_2SO_4 was used as a standard and was assumed to have an absolute quantum yield of 0.70 (Scott et al., 1970) at 25 °C. The area of the emission spectrum was determined by cutting out and weighing the recorded spectrum. The absorbance of samples was kept below 0.05 (for a 1-cm path length) to minimize inner filter effects. The values of steady-state polarizations were calculated by the method of Azumi & McGlynn (1962) with excitation at 342 nm (5-cm bandwidth) and emission at 375 nm (8-nm bandwidth) for NPM derivatives or with excitation at 312 nm (5-nm bandwidth) and emission at 368 nm (8-nm bandwidth) for NBPM derivatives. The steady-state fluorescence polarization, P, is defined as

$$P = (I_{vy} - fI_{vh})/(I_{vy} + fI_{vh}) \quad (3)$$

where I_{vy} and I_{vh} are the relative fluorescence intensities with the excitation and emission polarizers in parallel and perpendicular orientations, respectively, and f is the correction factor for photomultiplier sensitivity. The emission anisotropy, r, was calculated from the polarization value by using the relationship $r = 2p/(3 - p)$, where p is the polarization value and r is the emission anisotropy. The limiting anisotropy, r_0, was determined by plotting the reciprocal of the emission anisotropy, r, vs. T/η for a series of solutions containing 0–50% (w/v) glycerol in Hepes buffer. T is the absolute temperature and η is the solvent viscosity (Perrin, 1926).

The efficiency of energy transfer from enzyme labeled with NPM, NBPM, DDPM, or NBD was measured by comparison of the fluorescence of enzyme containing only the energy donor with that of enzyme containing both donor and acceptor. The protein concentration was similar for both samples.

Data Analysis. The theory of energy transfer between fluorescing and absorbing species was developed by Förster (1959, 1965). The energy transfer is related to the distance between the donor–acceptor pair according to eq 4. In this

$$R = R_0(E^{-1} - 1)^{1/6} \quad (4)$$

equation E is the efficiency of energy transfer (defined below) and R is the distance between donor and acceptor. R_0, the distance for 50% energy transfer, may be calculated from the known properties of the energy donor and acceptor by eq 5.

$$R_0 = (9.785 \times 10^3)(J\kappa^2 Q_D n^{-4})^{1/6} \quad (5)$$

In this equation, n is the refractive index of the medium (1.33 for water), Q_D is the quantum yield of the donor, κ^2 is an orientation factor dependent on the relative orientations of the donor dipole and the acceptor dipole, and J is the normalized spectral overlap integral. The spectral overlap integral is a measure of how well the fluorescence emission of the donor overlaps the absorption spectrum of the acceptor and is calculated from eq 6, where F is the donor fluorescence, ϵ is the

$$J = \int F(\nu)\epsilon(\nu)\nu^{-4}\, d\nu / \int F(\nu)\, d\nu \quad (6)$$

molar extinction coefficient of the acceptor, and ν is the frequency. The major source of error in the calculation of R_0 is the value of κ^2, which cannot be directly measured. This value in general can range from 0 to 4 with a value of $^2/_3$ for

Table I: Properties of Modified Carbamoyl-phosphate Synthetase[a]

derivative[b]	% act.	% act. regained	polarization
control	100		
NPM_1	96.2		0.185
$DDPM_1$	95.3		
$NBPM_1$	96.8		0.364
CPM_1	94.3		
$DTNB_1$			
NPM_1DDPM_2	5.1		0.185
NPM_1DTNB_2	5.0		
$NPM_1DTNB_2DDPM_3$	2.7		
NPM_1DDPM_3	7.0	4.3	0.186
$DDPM_1NPM_2$	5.6		0.189
$DDPM_1DTNB_2$	5.4		
$DDPM_1DTNB_2NPM_3$	1.7		
$DDPM_1NPM_3$	7.1	5.4	0.160
$DTNB_1NPM_2$	8.7		
NPM_2	11.7	3.0	0.190
$DTNB_1NPM_2DDPM_3$	3.2		
NPM_2DDPM_3	6.2	3.0	0.191
$DTNB_1DDPM_2$	8.3		
$DDPM_2$	11.5	3.2	
$DTNB_1DDPM_2NPM_3$	1.9		
$DDPM_2NPM_3$	4.9	3.0	0.158
$DTNB_1DTNB_1$	6.6		
$DTNB_1DTNB_2NPM_3$	1.7		
NPM_3	7.2	5.5	0.159
$NBPM_1DDPM_2$	5.8		0.364

[a] All assays were conducted at pH 8.2, $T = 37$ °C, as described under Materials and Methods. [b] The subscripts refer to covalent derivatives at sulfhydryl sites 1, 2, and 3 as described under Materials and Methods.

random orientation between the energy acceptor and donor. However, using emission anisotropy measurements one can determine the minimum and maximum values of κ^2 by the method developed by Dale & Eisinger (1974). Polarization measurements were conducted as explained above. The efficiency of energy transfer (Förster, 1959, 1965) is defined as

$$E = 1 - Q_{D/A}/Q_D \quad (7)$$

where $Q_{D/A}$ is the quantum yield of the donor in the presence of the acceptor, and Q_D is the quantum yield of the donor in the absence of transfer.

Results

Labeling the Various Sulfhydryl Sites of Carbamoyl-phosphate Synthetase. Carbamoyl-phosphate synthetase was labeled (see Methods) with various NEM derivatives and DTNB. These modified enzymes are listed in Table I and the subscripts refer to which sulfhydryl was modified by each compound.

Labeling the first sulfhydryl with any NEM derivative or DTNB results in very little loss of activity (3–6%). When the first sulfhydryl was labeled with NPM and the second with either DDPM or DTNB, only 5% of the original activity remained. When the CPS–NPM_1DTNB_2 derivative was further reacted with DDPM, another 2% of the activity was lost. Removal of the DTNB from this triply labeled enzyme with DTE resulted in a regain of about 4% of the activity.

A loss of 95% of the original activity was also found when the first sulfhydryl was labeled DDPM and the second with either NPM or DTNB. Reaction of the CPS–$DDPM_1DTNB_2$-derivatized enzyme with NPM produced another 3% loss in activity. A 5% increase in activity followed as a result of removal of the DTNB which was at the second sulfhydryl site.

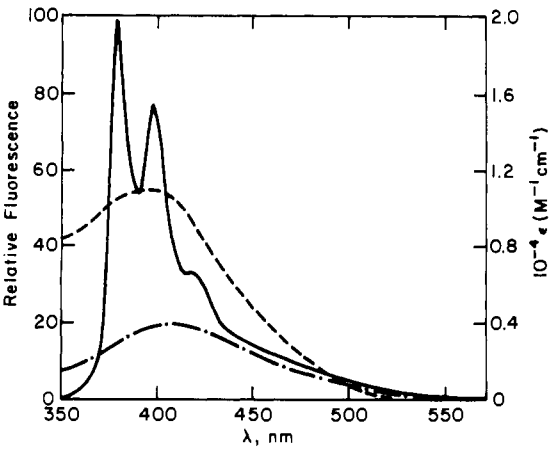

FIGURE 1: Spectral overlap of the NPM fluorescence emission spectrum (342-nm excitation) of the CPS–$NPM_{1,2,3}$ derivatives (—) with the extinction coefficients (ϵ) of NBD (---) and DDPM (–·–).

When DTNB was used to label the first site and either NPM or DDPM labeled the second site, 8–9% of the original activity remained. Removal of the DTNB from the first site with either NPM or DDPM at the second site resulted in a 3% regain in activity. Labeling of CPS–$DTNB_1NPM_2$ with DDPM resulted in a further 5% loss of activity, while labeling of CPS–$DTNB_1DDPM_2$ with NPM resulted in a 6% activity loss. When the DTNB was removed from both of these adducts, there was a 3% regain of activity.

Enzyme doubly modified with DTNB produced a 93% loss of activity which was followed by a further 5% activity loss when site three was labeled with NPM. Removal of DTNB from the first two sulfhydryls resulted in a regain of almost 6% of the original activity.

Finally, labeling the first site with NBPM and the second with DDPM gave an overall 94% loss of activity.

The polarization values of several samples are also listed in Table I. When NPM was at the first site, the polarization value was 0.185, which was similar to the value of 0.190 obtained with NPM at the second site. However, with NPM at the third site a lower value of 0.159 was obtained. The polarization value of the NBPM derivative, 0.364, was much higher than that for any of the enzyme derivatives with NPM.

Energy Transfer Experiments. The fluorescence emission spectra of the NPM or NBPM CPS derivatives remained unchanged whether sulfhydryl site 1, 2, or 3 was labeled. The spectral overlaps for NPM with DDPM and NBD and for NBPM with CrATP and $Co(NH_3)_4ATP$ are given in Figures 1 and 2, respectively. All other spectral overlaps are contained in the supplementary material (see paragraph at end of paper regarding supplementary material).

Experiments were conducted to measure energy transfer between several sites. Table II lists the efficiency of energy transfer, E, for various combinations of fluorescent donors and acceptors covalently bound to sulfhydryl sites 1, 2, and 3 in several combinations.

Quantum yields and overlap integrals are included in Table II along with calculated values of R_0 (assuming κ^2 to be $^2/_3$) for the various sulfhydryl–sulfhydryl donor–acceptor pairs.

The calculated distance between SH_1 and SH_2 using various pairs of labels was in the range 27–33 Å and was similar to the calculated distance between SH_1 and SH_3. However, the calculated distance between SH_2 and SH_3 was shorter (~22 Å).

To test for possible discrepancies in energy transfer efficiency due to the nature of the label at each sulfhydryl site, the labels were reversed between sites 1 and 2. When NPM

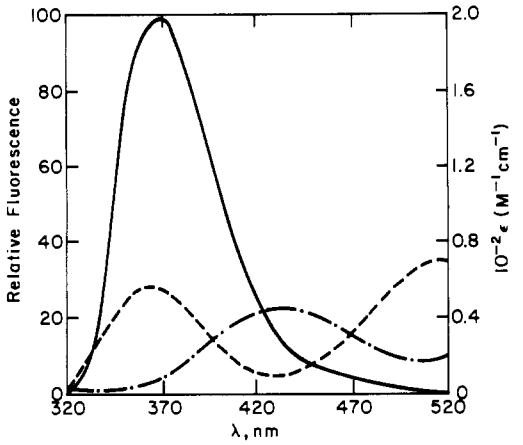

FIGURE 2: Spectral overlap of the NBPM fluorescence emission spectrum (312-nm excitation) of the CPS-NBPM$_1$ derivative (—) with the extinction coefficients (ϵ) of $Co(NH_3)_4ATP$ (---) and CrATP (-·-).

Table II: Energy Transfer Parameters and Data for Various Derivatives

donor	acceptor	Q_D	$Q_{D,A}$	E_c[a]	J	R_0 (Å)[b]	R (Å)
			SH_1-SH_2 Distance				
NPM_1	$DDPM_2$	0.242	0.146	0.401	0.576×10^{-14}	25.3	27
NPM_2	$DDPM_1$	0.238	0.152	0.366	0.576×10^{-14}	25.2	28
NPM_1	NBD_2	0.242	0.158	0.424	0.221×10^{-13}	31.6	33
$NBPM_1$	$DDPM_2$	0.300	0.191	0.367	0.407×10^{-14}	24.7	27
$NBPM_1$	NBD_2	0.300	0.201	0.407	0.168×10^{-13}	31.3	33
			SH_1-SH_3 Distance				
NPM_1	$DDPM_3$	0.242	0.131	0.459	0.576×10^{-14}	25.3	26
NPM_3	$DDPM_1$	0.277	0.165	0.424	0.576×10^{-14}	25.9	27
			SH_2-SH_3 Distance				
NPM_2	$DDPM_3$	0.238	0.057	0.765	0.576×10^{-14}	25.2	21
NPM_3	$DDPM_2$	0.277	0.069	0.755	0.576×10^{-14}	25.9	22

[a] The efficiency was corrected for the acceptor stoichiometry. [b] R_0 was calculated by using $^2/_3$ for κ^2.

was used as the donor at either the first or the second site with DDPM as the acceptor, similar distances were obtained in both cases. The same value was obtained with NBPM as the donor at the first site and DDPM as the acceptor at the second site. When NBD was the acceptor with either NPM or NBPM as the doner, a value of 33 Å was obtained for the distance. The values of 28 and 33 Å are essentially the same within experimental error for the different acceptors used, and the difference is similar to results obtained when the same donor–acceptor pairs were used in another enzyme system (Craig & Hammes, 1980).

When the enzyme was labeled with NEM at the first site and NPM at the second site, a quantum yield of 0.202 was obtained. However, when the first site was labeled with DTNB and the second with NPM followed by removal of the DTNB, the quantum yield was 0.237, demonstrating that modification of the first sulfhydryl produces a small conformational change resulting in partial quenching of the donor signal.

Active Site to Sulfhydryl Site Distance Determinations. The bidentate β,γ-CrATP and β,γ-bidentate $Co(NH_3)_4ATP$ were found to be good competitive inhibitors vs. MgATP at pH 6.5 in 50 mM Pipes buffer (Raushel et al., 1979). Thus, they could be used as acceptors because their absorption spectra overlap the fluorescence emission of the donors. Table III presents the energy transfer parameters for various sulfhydryl derivatives. Energy transfer efficiencies for combinations of various donors and acceptors are also listed in this

Table III: Energy Transfer Data and Parameters for Carbamoyl-phosphate Synthetase Complexes

donor	acceptor[a]	Q	E[b]	J	R_0 ($^2/_3$)	$R(^2/_3)$[c]
NPM_1	$Co(NH_3)_4ATP$	0.242	0.05	0.975×10^{-16}	12.7	≥21
NPM_1	CrATP	0.242	0.05	0.860×10^{-16}	12.5	≥21
$NBPM_1$	$Co(NH_3)_4ATP$	0.300	0.05	0.889×10^{-16}	13.2	≥21
$NBPM_1$	CrATP	0.300	0.05	0.418×10^{-16}	11.5	≥19
CPM_1	$Co(NH_3)_4ATP$	0.332	0.05	0.196×10^{-15}	15.2	≥25
CPM_1	CrATP	0.332	0.05	0.155×10^{-15}	14.6	≥24
NPM_2	$Co(NH_3)_4ATP$	0.238	0.05	0.975×10^{-16}	12.8	≥21
NPM_2	CrATP	0.238	0.05	0.860×10^{-16}	12.5	≥20
NPM_3	$Co(NH_3)_4ATP$	0.277	0.05	0.975×10^{-16}	13.1	≥21
NPM_3	CrATP	0.277	0.05	0.860×10^{-16}	12.8	≥21

[a] All titrations were carried out in Pipes buffer at pH 6.5, T = 25 °C. [b] No measurable difference in quenching between MgATP, $Co(NH_3)_4ATP$, or CrATP was observed and thus an efficiency of 0.05 was used. [c] With an efficiency of 0.05, only a limit of the distance could be obtained.

Table IV: Analysis of the Orientation Factor κ^2 for CPS-NPM_1DDPM_2

parameter	symbol	donor (NPM)	acceptor (DDPM)
emission anisotropy	r	0.131	0.276
limiting anisotropy[a]	r_0	0.215	0.296
dynamic depolarization factor[b]	$\langle d' \rangle$	0.538	0.740
cone half-angle for volume (deg)	Ψ	36	25.5
cone half-angle for surface (deg)	Ψ	26.5	18
range of κ^2	$0.18 \leq \kappa^2 \leq 2.94$		
range of R_0 (Å)	$20.3 \leq R_0 \leq 32.4$		(25.3)[c]
range of R (Å)	$22 \leq R \leq 34$		(27)[c]

[a] The limiting anisotropy, r_0, is obtained by measuring $1/r$ as a function of the viscosity which was varied by the addition of glycerol. [b] The dynamic depolarization factor is defined by $\langle d' \rangle = r_0/0.4$. [c] Values in parentheses are obtained by using $^2/_3$ for κ^2.

table. Since the R_0 values all lie in the range 11–15 Å, distances of greater than 20 Å between donor and acceptor will produce <5% change in energy transfer efficiency. This was found for all combinations of nucleotides and fluorescent labels used with carbamoyl-phosphate synthetase.

Fluorescence Anisotropy Measurements. The range of values for the geometrical factor κ^2 can be limited by the use of fluorescence anisotropy measurements according to Dale & Eisinger (1974). The extreme estimates of κ^2 for one donor–acceptor pair, CPS-NPM_1DDPM_2, are summarized in Table IV. As shown, the half-angles of conical volume and surface within or upon which the chromophore has rotational freedom were determined. Values of 36° and 25.5° were obtained for NPM and DDPM, respectively, for the half-angles of conical volume. Likewise, values of 26.5° and 18° were obtained for NPM and DDPM for the half-angles of conical surface. Thereafter, the range of values was computed from the graphical analysis of Dale & Eisinger (1974). The geometrical factor is restricted to values between 0.18 and 2.94, resulting in a range of 19.0–30.2 Å for the critical distance, R_0, for this donor–acceptor pair. The actual SH_1 to SH_2 distance, therefore, lies between the extreme limits 22 and 34 Å, as compared to the $R(^2/_3)$ value of 27 Å.

The same types of limits were determined for all the calculated distances and results are contained in the supplementary material.

Effect of Substrates and Modifiers on Distance Determinations. The results of titrations with various donors and acceptors present are given in Table V. Only a slight change

Table V: Titration of Various Derivatives of Carbamoyl-phosphate Synthetase with Substrates and Modifiers[a]

donor	acceptor	ornithine	glutamine	$MgCl_2$	ATP	$KHCO_3$	IMP	UMP
NPM_1	$DDPM_2$	NC	NC	NC	NC	NC	SC − 7%	SC − 7%
NPM_1	$DDPM_3$	NC	NC	NC	NC	NC	SC − 6%	SC − 5%
NPM_2	$DDPM_3$	NC	SC + 4%	NC	SC + 4%	NC	NC	NC
$NPM_{1,2,\text{or }3}$	$Co(NH_3)_4ATP$	NC	NC	NC	NC	NC	NC	NC
$NPM_{1,2,\text{or }3}$	CrATP	NC	NC	NC	NC	NC	NC	NC

[a] NC = no change. SC = slight change. (+) = increase in fluorescence. (−) = decrease in fluorescence.

in conformation occurred when IMP and UMP were added with NPM_1 as the donor and either $DDPM_2$ or $DDPM_3$ as the acceptor. When NPM_3 was the donor and $DDPM_2$ the acceptor, a slight change was observed when either glutamine or ATP was added. Titration with the ATP analogues present caused no observable changes.

Discussion

Carbamoyl-phosphate synthetase from *E. coli* has at least 10 separate ligand binding sites for substrates, activators, and allosteric modifiers. The goal of this work and that presented in the previous paper (Raushel et al., 1983) was to elucidate the spatial relationships among these various sites.

Based on the work of Matthews & Anderson (1972) and Trotta et al., (1971), it was shown that three sulfhydryls could be specifically labeled with NEM or DTNB. Sites 1 and 3 are on the large subunit and site 2 is on the small subunit. Earlier work in our laboratory (Raushel et al., 1979) demonstrated that each sulfhydryl could also be labeled with nitroxide maleimide derivatives. In the present work, we demonstrated that fluorescent maleimide derivatives can be used to selectively modify the sulfhydryls. With specific fluorescent labels we used fluorescence energy transfer to calculate the probe to probe distances when each fluorescent probe was attached to a sulfhydryl group. Using the appropriate donor–acceptor pairs, we also computed probe (on a sulfhydryl) to substrate distances with the same experimental approach.

The results in Table I show that the first sulfhydryl can be selectively labeled by DTNB or four other maleimide derivatives. The stoichiometry of labeling with any of these derivatives was between 0.9 and 1.0. With this stoichiometry we could then selectively label a second site and have two different labels with one donor and one acceptor in any order. Since the calculated distances are in the range of 20–35 Å, a slight amount of labeling (~5%) of "another" site while one "specific" site was labeled is inconsequential.

From Table II, when the donor–acceptor pair NPM–DDPM was used, the same probe to probe distance was obtained, indicating that no major protein conformational change took place with NPM at either the first or second site. The same distance was also obtained with a different donor, NBPM, and with a different acceptor, NBD.

Modification does produce a conformational change affecting the overall reaction, i.e., the formation of carbamoyl phosphate. However, Matthews & Anderson (1972) found that the partial reverse reaction (formation of ATP from carbamoyl phosphate and ADP) is *stimulated* 2-fold when the third sulfhydryl is modified by NEM. This result demonstrates that subtle changes in the active site occur upon modification of the sulfhydryls, leading to an alteration of some of the catalytic properties of the enzyme. Importantly, since enzyme activity remains, the data obtained with fluorescent probes do report changes that nearly reflect the catalytically competent form of the enzyme.

When NEM was used to derivatize a sulfhydryl group with various donor–acceptor pairs on the other two sulfhydryls, a slight quenching of the donor signal resulted, so NEM alone could not be used as a blank. This problem was largely overcome by derivatizing one sulfhydryl with DTNB and the other two sulfhydryls with various donor–acceptor pairs. Removal of DTNB in all cases provided enzyme derivatives free of interferences from additional quenching mechanisms. As is shown in Table II, the same value was obtained for the SH_1–SH_3 distance whether the donor, NPM, was at the first or the third site. Also, when the SH_2–SH_3 distance was determined, the same value was obtained whether the NPM was at the second or third site.

In the determination of the spatial relationship between probes attached to the three sulfhydryl residues and the active site using ATP analogues, only a limit of the distance, ≥21 Å, could be obtained. The same limit was obtained from EPR experiments in our laboratory (Raushel et al., 1979) using spin-labeled NEM derivatives and Mn–nucleotides.

The dipole orientation factor, κ^2, is the least certain parameter in calculating the critical transfer distance, R_0. Although theoretically κ^2 can range from 0 to 4, the extreme values require very rigid orientations. If both the donor and acceptor are tumbling rapidly and are free to assume any orientation, then κ^2 equals $^2/_3$ (Förster, 1959). If only the donor is free to rotate, then κ^2 can vary from $^1/_3$ to $^4/_3$ (Wu & Stryer, 1972). The validity of assuming a value of $^2/_3$ has been discussed often (Stryer, 1978; Hillel & Wu, 1976; Matsumoto & Hammes, 1975; Dale & Eisinger, 1974). In our study we used fluorescence polarization measurements to better define a range for κ^2.

The steady-state fluorescence polarization of fluorescence labels reflects their rotational motion (Webster, 1953). The low polarization of the NPM derivatives (0.157–0.190; 0.5 for a rigidly held donor) indicates that the use of $\kappa^2 = {}^2/_3$ will not be seriously in error; a maximum uncertainty of 20% in R was estimated in Table IV (Dale & Eisinger, 1974), and the probability distribution for R is quite narrow (Hillel & Wu, 1976). Since free rotation of both the donor and acceptor cannot be assumed for this system, several sets of donor–acceptor pairs were used to measure the same distance (Table II). The good agreement between the distances measured with the different donor–acceptor pairs assuming $\kappa^2 = {}^2/_3$ suggests that κ^2 is close to this average value in all cases.

Because we can accurately measure the distance between different sites, substrates and modifiers of the enzyme were titrated with donors and acceptors present to observe any conformational changes that may occur. When the first and second sulfhydryls or the first and third sulfhydryls were derivatized, a slight change in the fluorescence was observed when IMP or UMP were added. A slight change was also observed when the second and third sites were derivatized and either ATP or glutamine was added. This change was observed in earlier EPR experiments in our laboratory (Raushel et al., 1979) when the second sulfhydryl was derivatized with a spin-label and ATP or glutamine was added. These changes are probably due to the fact that the second sulfhydryl is located on the small (glutamine binding) subunit (M_r 48 000).

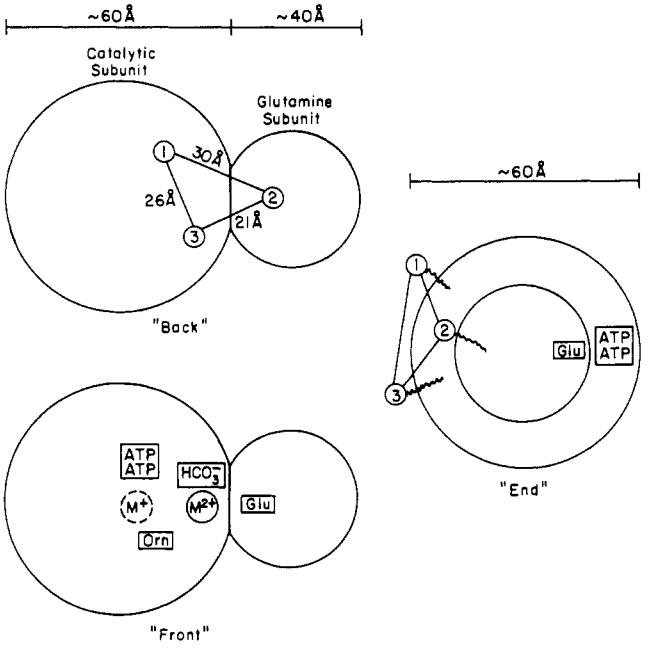

FIGURE 3: Schematic drawing of carbamoyl-phosphate synthetase showing the dimensions among the various sulfhydryl sites and the active site.

This sulfhydryl group is totally unreactive in the absence of MgATP plus HCO_3^-. No changes were observed when either site 1, 2, or 3 was labeled with a donor and the ATP analogues were used as acceptors.

In summary, three different sulfhydryl groups of *E. coli* carbamoyl-phosphate synthetase have been modified with various fluorescent maleimide probes. By the use of fluorescence energy transfer, it has been demonstrated that all three labeled sulfhydryls are ≥21 Å from the ATP sites. The distance between the first and second sulfhydryls, 27 Å, was found to be approximately equal to the distance between the first and the third sulfhydryls. The calculated distance between the second and third sulfhydryls was 21 Å. A schematic drawing of these distance relationships and those from the previous paper is given in Figure 3. The dimensions for each subunit are estimates based on the molecular weight for each subunit. The best model that fits all the distance data is one in which the three sulfhydryl groups are on one "side" of the dimer while the active site is on the opposite "side". Future experiments will focus on using the spin-labeled derivatives as paramagnetic probes and other fluorescent probes to obtain additional spatial relationships on carbamoyl-phosphate synthetase.

Supplementary Material Available

Figures showing overlaps of the fluorescence emission of NPM, NBPM, and CPM with various acceptors and a table giving orientation factor κ^2 analysis data (4 pages). Ordering information is given on any current masthead page.

Registry No. CPS, 37233-48-0; ATP, 56-65-5; IMP, 131-99-7; UMP, 58-97-9; glutamine, 56-85-9.

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