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THE USE OF SPECIFIC LYSINE MODIFICATIONS TO LOCATE THE REACTION SITE OF CYTOCHROME *c* WITH SULFITE OXIDASE

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Summary

The reduction of cytochrome *c* by beef liver sulfite oxidase was found to be strongly inhibited by high ionic strength, indicating the importance of electrostatic interactions to the reaction. The reaction rates of sulfite oxidase with singly trifluoroacetylated or trifluoromethylphenylcarbamylated cytochrome *c* derivatives were studied to determine the role of individual lysines in the reaction. The reaction rate was decreased by modification of the lysines immediately surrounding the heme crevice, the decreases following the order: Lys 13 > Lys 25 ≃ Lys 79 ≈ Lys 87 > Lys 8 ≈ Lys 27 ≈ Lys 72. Modification of lysines 22, 55, 88, 99, and 100 had no effect on the reaction rate. These results indicate that the interaction site on cytochrome *c* for sulfite oxidase is at the heme crevice region, and overlaps considerable with that for cytochrome oxidase.

Introduction

The terminal step in the degradation of sulfur-amino acids is the oxidation of sulfite to sulfate catalyzed by sulfite oxidase [1]. Oshino and Chance [2] have shown that sulfite oxidase located in the intermembrane space of liver mitochondria transfers electrons from sulfite to cytochrome *c* located on the outer side of the inner mitochondrial membrane. The ATP/O ratio for sulfite respiration was half that observed for succinate respiration, indicating electron flow through the cytochrome oxidase site of the respiratory chain. Sulfite oxidase

Abbreviations: $CF_3PhNHCO$-, *m*-trifluoromethylphenylcarbamoyl; Mops, 3-(*N*-morpholino)propanesulfonic acid; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

from rat liver is a dimer of molecular weight 120 000 consisting of two identical polypeptide chains [3]. The molybdenum and heme prosthetic groups are thought to be present in separate domains since treatment of the native enzyme with trypsin yields a heme containing monomer of molecular weight 10 000 and a molybdenum containing dimer of molecular weight 100 000. The tryptic heme domain of sulfite oxidase is very similar to the heme domains of cytochrome b_5 and flavocytochrome b_2 in terms of molecular weight, amino acid composition, and absorption spectra [3,4]. However, Guiard and Lederer [4] found that the heme domain of chicken liver sulfite oxidase was not cross reactive with antibodies elicited against calf liver microsomal cytochrome b_5, and was only 12% as active towards cytochrome b_5 reductase as cytochrome b_5 itself. The same authors [5] have recently reported the amino acid sequence of chicken liver sulfite oxidase heme domain. Although there are sufficient similarities with the sequence of cytochrome b_5 from chicken liver [6,7] to support their hypothesis of a common evolutionary origin, there are also very significant differences. Both cytochromes are highly acidic and react rapidly with cytochrome *c*.

Recently, cytochrome *c* derivatives specifically modified at single lysine residues have been used to define the interaction domain on cytochrome *c* for cytochrome b_5 [8], cytochrome oxidase [9—12], cytochrome c_1 [13,14] and cytochrome *c* peroxidase [15]. In all cases the reaction site is located at the front of the cytochrome *c* molecule, and the positive charges on the five or six lysine groups immediately surrounding the heme crevice are involved in complementary charge interactions with negatively charged carboxyl groups on the other proteins. In the present paper we use these techniques to define the interaction domain on cytochrome *c* for sulfite oxidase. Dethmers et al. [16] have also studied this reaction using mono-carboxydinitrophenyl lysine derivatives of cytochrome *c* and found that the reaction site is at the heme crevice region.

Materials and Methods

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. Tris (ultra pure) was obtained from Schwartz/Mann. Sodium sulfite, analyzed reagent, was obtained from Baker Chemical Co. The specifically trifluoroacetylated (CF_3CO-) derivatives were prepared according to the procedures of Staudenmayer et al. [11,12] and Smith et al. [17], while the trifluoromethylphenylcarbamoyl ($CF_3PhNHCO$-) derivatives were prepared by the procedure of Smith et al. [10]. All derivatives were chromatographed a final time on a 1.5 × 10 cm column of Whatman CM32 carboxymethyl-cellulose eluted with 0.08 M phosphate buffer (pH 6.0). Fractions were analyzed by ^{19}F-NMR techniques [11] to determine their purity before they were pooled. The derivatives were never lyophilized at any point in the purification.

Sulfite oxidase assays. The sulfite oxidase was extracted from beef livers according to the procedure used by Johnson and Rajagopalan [18] for rat livers. This procedure yielded a partially purified preparation for which the 413/280 nm absorbance ratio was 0.25. Assays were conducted by adding 20 μl sulfite oxidase to a cuvette containing 0.5 to 5 μM ferricytochrome *c*, 0.10 M Tris-HCl, pH 7.5 and 0.5 mM sodium sulfite. The ionic strength was

adjusted by the addition of sodium chloride. The rate of reduction of cytochrome *c* following addition of sulfite oxidase was followed at 420 or 550 nm on a Varian Techtron model 635 spectrophotometer.

Results

The reduction of cytochrome *c* by sulfite oxidase has been shown to occur by a ping-pong mechanism [19]. Since it is quite likely that there is a slow and potentially rate-limiting step prior to the actual reduction of cytochrome *c*, the kinetic parameter which is most clearly a function of the cytochrome *c* step is V_{max}/K_m. At low cytochrome *c* concentrations the reaction was found to be first order in cytochrome *c*, with a rate constant equal to V_{max}/K_m. Increasing ionic strength did not affect the apparent maximum velocity of the reaction, but caused a large decrease in V_{max}/K_m, indicating electrostatic interactions were important to the reaction (Fig. 1A). It was found that $\ln(V_{max}/K_m)$ decreased almost linearly as $\sqrt{I}$ increased up to 0.6, as shown in Fig. 1B. Specific modification of cytochrome *c* lysines with CF_3CO- or $CF_3PhNHCO$- had no effect on the apparent V_{max} of the reaction, but modification of lysines 8, 13, 25, 27, 72, 79, and 87 surrounding the heme crevice caused a significant decrease in V_{max}/K_m (Fig. 2, Table I). On the other hand, the reaction rate was completely unaffected by modification of lysines 22, 55, 88, 99 and 100 on the right side, bottom left, top left, and back of cytochrome *c*, respectively (Fig. 2, Table I). These results clearly indicate that the electrostatic interaction

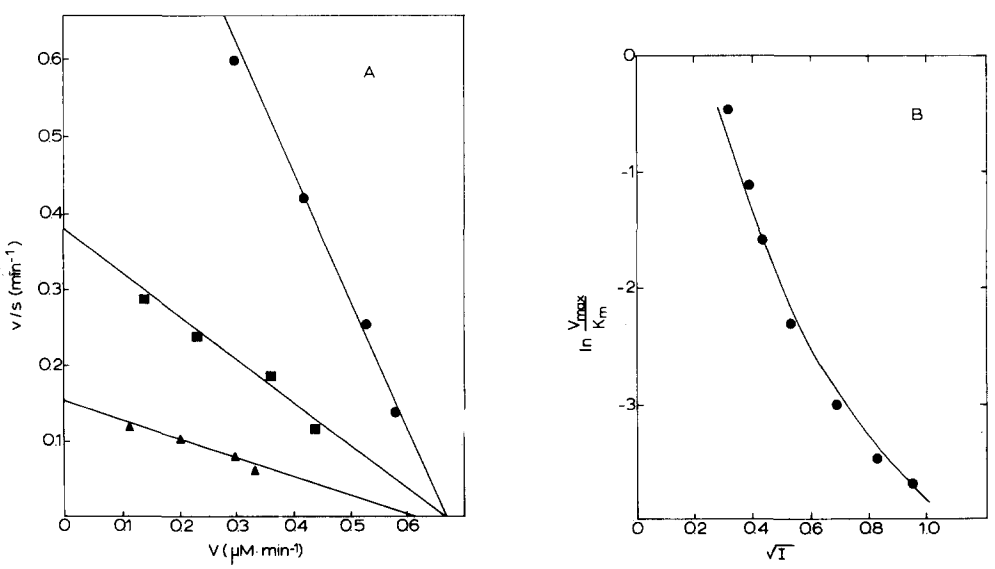

Fig. 1. Effect of ionic strength on the rate of the reaction between cytochrome *c* and sulfite oxidase at 25°C. The assay solution contained 0.05 M Tris-HCl, pH 7.5, NaCl to bring the total ionic strength to the indicated value, 0.4 mM sodium sulfite, and 3.0 nM sulfite oxidase. Cytochrome *c* concentrations are in μM, and the initial velocity, *v*, is in μM cytochrome *c* reduced per min. (A) Eadie-Hofstee plots at different ionic strengths: ●——●, 0.070; ■——■, 0.10; ▲——▲, 0.15. (B) Logarithmic dependence of (V_{max}/K_m) on the square root of ionic strength. The solid line was calculated from Eqn. 5 using a = 1.7 Å and r_i values shown in Table II.

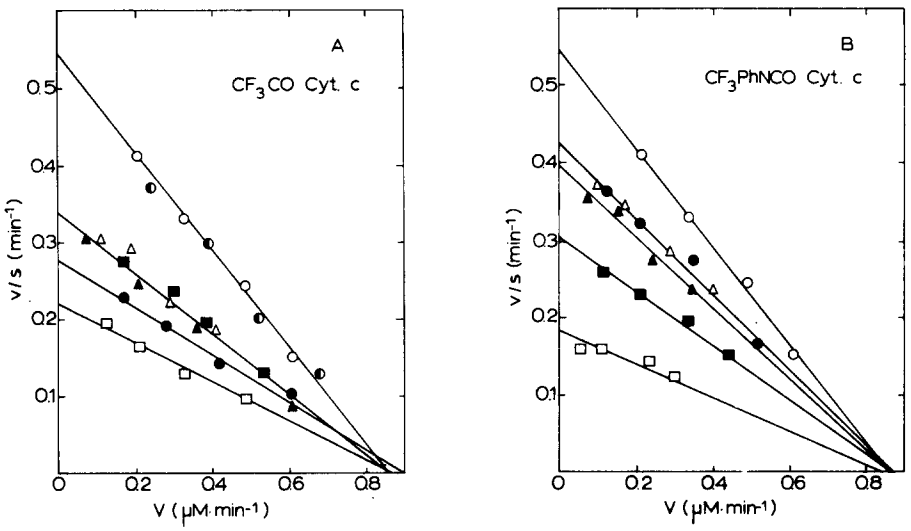

Fig. 2. Reaction rates of singly-labeled cytochrome *c* (Cyt *c*) derivatives with sulfite oxidase. The assay solution contained 0.1 M Tris-HCl, pH 7.5, 0.4 mM sodium sulfite, and 3.0 nM sulfite oxidase at 25°C. The cytochrome *c* concentration *s* is in μM, and the initial velocity *v* is in μM/min. (A) The symbols used for the trifluoroacetyl derivatives are: ○——○, native; ◐——◐, 88; △——△, 87; ■——■, 79; ▲——▲, 72; ●——●, 25; □——□, 13. (B) The symbols used for the $CF_3PhNHCO$-derivatives are: ○——○, native; △——△, 8; ●——●, 27; ▲——▲, 72; ■——■, 79; □——□, 13.

TABLE I

EFFECT OF SPECIFIC LYSINE MODIFICATIONS ON THE REDOX REACTIONS OF CYTOCHROME *c*

The relative reactions rates are presented as $Y = (V_{max}/K_m)$ native/(V_{max}/K_m) derivative. The sulfite oxidase reaction rates were measured at 25°C in 0.1 M Tris-HCl, pH 7.5, 0.4 mM sodium sulfite, and 3.0 nM sulfite oxidase. The cytochrome b_5 reaction rates were determined from the initial velocity of reduction of cytochrome *c* by calf liver microsomes in 50 μM NADH, 0.1 M Tris-HCl, pH 7.5, 25°C [8, 17]. The cytochrome c_1 reaction rates were determined from the initial velocity of reduction of cytochrome *c* by succinate-cytochrome *c* reductase at 25°C in 10 mM succinate, 0.2 M Tris-HCl, pH 7.5 [13, 17]. The cytochrome oxidase reaction rate was measured polarigraphically at 25°C in 10 mM ascorbate, 1.0 mM TMPD, 0.2 M sucrose, and 60 mM potassium Mops, pH 7.5 [10,17].

Derivative	Sulfite oxidase	Cytochrome b_5	Cytochrome c_1	Cytochrome oxidase
$CF_3PhNHCO$-Lys-8	1.3	1.4	1.7	2.4
CF_3CO-Lys-13	2.4	2.7	4.2	4.9
$CF_3PhNHCO$-Lys-13	2.9	2.7	3.8	6.9
CF_3CO-Lys-22	1.1	1.0	1.0	1.1
CF_3CO-Lys-25	2.0	1.8	0.94	2.7
$CF_3PhNHCO$-Lys-27	1.3	1.8	2.1	1.6
CF_3CO-Lys-55	1.1	1.0	0.97	1.0
CF_3CO-Lys-72	1.7	3.0	2.1	3.1
$CF_3PhNHCO$-Lys-72	1.3	2.5	2.1	2.7
CF_3CO-Lys-79	1.6	1.8	2.5	1.9
$CF_3PhNHCO$-Lys-79	1.6	1.9	2.3	1.8
CF_3CO-Lys-87	1.5	1.5	2.0	2.8
CF_3CO-Lys-88	1.0	1.0	1.5	1.4
CF_3CO-Lys-99	1.0	0.97	1.0	0.90
$CF_3PhNHCO$-Lys-100	1.0	1.0	1.0	0.94

between cytochrome *c* and sulfite oxide is dominated by complementary charge pair interactions between the lysine amino groups immediately surrounding the heme crevice of cytochrome *c* and negatively charged carboxylate groups on sulfite oxidase.

The effect of electrostatic interactions on the reaction rate can be described by the Marcus theory formalism as:

$$\ln k = \ln k_\infty - V/RT \tag{1}$$

where V is the electrostatic free energy of interaction between the two proteins in the activated complex and k_∞ is the rate constant at infinite ionic strength [20]. The most general expression for V is:

$$V = \sum_i V_i \; ; \quad V_i = \sum_j V_{ij} \tag{2}$$

where the first summation is over all charged groups i on protein A, the second summation is over all charged groups j on protein B, V_{ij} is the electrostatic free energy of interaction between charges i and j, and V_i is the energy of interaction of one group i on protein A with all charged groups on protein B. An estimate to the contribution a positively charged lysine amino group on cytochrome *c* makes to the electrostatic free energy of interaction with sulfite oxidase is given by:

$$V_i = -RT \ln y_i \tag{3}$$

where y_i is the ratio of the rate constant of native cytochrome *c* to that of a derivative modified at lysine i to change the charge from +1 to 0. This estimate might of course also include a contribution from steric interference of the modified group with the reaction. However, the close correspondence between

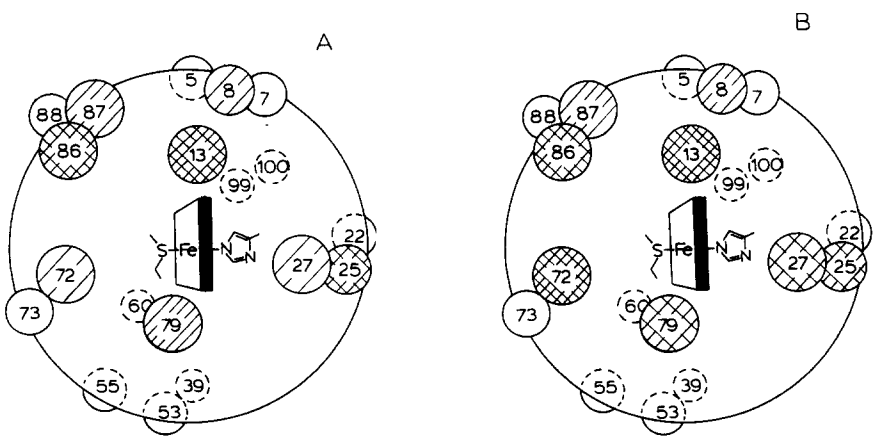

Fig. 3. A schematic diagram of horse heart cytochrome *c* viewed from the front of the heme crevice. The approximate positions of the β-carbon atoms of the lysine residues are indicated by closed and dashed circles for residues located towards the front and back of the molecule, respectively. The electrostatic free energy contribution of lysine *i*, V_i, calculated from Eqn. 3 is indicated by the number of diagonal hatch marks in the circle, with 0.05 kcal/mol per hatch mark. The V_i values for lysines 5, 7, 39, 53, 60, 73, and 86 were estimated by comparison with the values of adjacent lysines. (A) The interaction domain on cytochrome *c* for sulfite oxidase. (B) The interaction domain for cytochrome b_5.

the y_i values of the bulky $CF_3PhNHCO$-derivative and the CF_3CO-derivative at lysines 13, 72 and 79 indicate that such steric effects are small compared to the electrostatic interaction (Table I). Fig. 3 shows graphically the best estimates of V_i calculated from the smallest y_i value of the two types of derivative. The V_i values of lysines for which no derivative was available were estimated to be intermediate between the V_i values of adjacent lysines. The effect of specific modification of cytochrome *c* lysines on the reactions with cytochrome b_5, cytochrome c_1 and cytochrome oxidase are also shown in Table I and Fig. 3. The interaction domain on cytochrome *c* is very similar for all four proteins, but lysines 27 and 72 appear to be somewhat less important to the reaction with sulfite oxidase than to the other three reactions.

Salemme [21] has recently proposed a theoretical model for the activated complex between cytochrome *c* and cytochrome b_5 in which lysines 13, 27, 72, and 79 surrounding the heme crevice of cytochrome *c* form specific complementary charge pair interactions with the cytochrome b_5 carboxylate groups of Glu 52, Glu 48, Asp 64, and the heme propionate, respectively. A very significant feature of this model is that the distance between an amino group and a carboxylate group within a complementary charge pair interaction is about 3 Å, while the distance between this amino group and any other charged group is greater than 10 Å. To obtain a simplified expression for the electrostatic free energy of activation of cytochrome *c* reactions at ionic strengths above 0.1 M we shall assume that the total electrostatic interaction is dominated by a set of n complementary charge pairs each with charges $Z_i = -Z_j = 1$, and separation distance r_{ij}. The different charge pairs will be assumed to be separated from each other by at least 10 Å, and all interaction energies V_{ij} with r_{ij} greater than 10 Å will be neglected. Each complementary charge pair interaction will be estimated from Debye's formula [22]:

$$V_{ij} = 2.1175\left(\frac{e^{\kappa a_i}}{1+\kappa a_i} + \frac{e^{\kappa a_j}}{1+\kappa a_j}\right)\frac{Z_i Z_i\, e^{-\kappa r_{ij}}}{r_{ij}} \qquad (4)$$

where a_i is the effective radius of group *i*, $\kappa = 0.329\sqrt{I}$ Å^{-1}, *I* is the ionic strength, and the numerical constants are appropriate for aqueous solutions at

TABLE II

ELECTROSTATIC FREE ENERGY OF INTERACTION BETWEEN CYTOCHROME *c* LYSINES AND SULFITE OXIDASE

V_i was calculated from y_i using Eqn. 3 as described in the text. The ionic strength was 0.1 M. The value shown for lysine 86 was assumed by comparison with other adjacent lysines for which derivatives were available. r_i was calculated from the corresponding V_i values using Eqn. 4.

Lysine	$-V_i$ (kcal/mol)	r_i (Å)
8	0.15	10.0
13	0.53	4.8
25	0.41	5.8
27	0.15	10.0
72	0.15	10.0
79	0.28	7.2
86	0.50	5.0
87	0.24	8.0

25°C. Eqn. 4 assumes that each charge is fully solvated with electrolyte. Substitution into Eqns. 2 and 1 yields:

$$\ln k = \ln k_\infty + \sum_{i=1}^{n} \frac{7.152\, e^{\kappa(a-r_i)}}{r_i(1+\kappa a)} \tag{5}$$

where it was assumed that $a_i = a_j = a$.

In order to test this model we have used the V_i values obtained from the specific lysine modification studies to calculate from Eqn. 4 the distance r_{ij} between lysine i and a carboxylate group assumed to be complementary to it on sulfite oxidase (Table II). The effective radius a was assumed to be 1.7 Å. These r_{ij} values ranged from 4.8 Å for lysine 13 with a V_i value of −0.53 kcal/mol to 10 Å for lysines 8, 27, and 72, with V_i values of 0.15 kcal/mol. Lysine 86 was assumed to have r_{ij} = 5.0 Å. Eqn. 5 was then used to calculate the ionic strength dependence of the reaction rates, with good agreement between theory and experiment as shown in Fig. 1B. There are no charged groups on cytochrome *c* other than the ones shown in Table II that are likely to be closer than 10 Å to sulfite oxidase.

Discussion

The effect of specific modification of cytochrome *c* lysines on the reaction with sulfite oxidase clearly shows that the interaction domain involves lysines 8, 13, 25, 27, 72, 79, 86, and 87 immediately surrounding the heme crevice. The CF_3CO- and $CF_3PhNHCO$-derivatives are well suited for evaluating the importance of individual lysine amino groups to the electrostatic interaction, since the overall protein conformation and heme environment is not affected by the modification. We have previously shown that these derivatives have the same redox potential, visible absorption spectrum, optical rotatory dispersion spectrum and proton magnetic resonance spectrum as native cytochrome *c* [10—12]. These techniques do not rule out minor local conformational changes in the vicinity of the modified lysine which could interfere with the reaction, but the close correspondence between the y_i values of the bulky $CF_3PhNHCO$- and the CF_3CO-derivatives at several of the important lysines indicates such steric effects are relatively small compared to the electrostatic interaction. The accuracy of the V_i estimates is also supported by the good agreement between Eqn. 5 and the ionic strength dependence of the reaction rate when the parameters were based on the specific lysine modification results. It should be emphasized that Eqn. 5 is only applicable to reactions between proteins that have evolved a set of complementary charge interactions that dominate the total electrostatic interaction. Our neglect of interactions between charges separated by more than 10 Å is supported by the specific lysine modification studies which show that lysines slightly removed from the interaction domain, such as 88, make negligible contributions to the electrostatic interaction (Fig. 3). Also, Eqn. 4 predicts a very rapid decrease in V_{ij} as r_{ij} is increased: at ionic strengths above 0.1 M it is less than one-eighth as strong at r_{ij} = 10 Å as at 3.4 Å (Table II). At lower ionic strengths below 0.02 M, however, the long range interactions become more important, and models assuming uniform charge distributions on each protein will probably be applicable [20,23—25]. We have used Eqn. 4 at

ionic strengths considerably higher than those for which the assumptions used to derive the Debye-Hückel theory are strictly valid. However, Pitzer [26] has recently reviewed the application of rigorous Monte Carlo methods to monovalent electrolytes, and found excellent agreement with the exponential Debye-Hückel expression upon which Eqn. 4 is based at ionic strengths up to 0.42 M. Furthermore, Perlmutter-Haymann and Weismann [27] have studied the reaction between $CO(NH_3)_5Br^{2+}$ and OH^- in the presence of nine different monovalent electrolytes at ionic strengths up to 1.5 M, and found excellent agreement with the Debye-Hückel expression over the entire range.

It is of interest to compare the interaction domains for sulfite oxidase and cytochrome b_5, since it has been suggested that the heme peptides of the two proteins are evolutionarily related [3,4]. Lysines 13, 25, 79, and 87 appear to be equally important in both reactions, but lysines 27 and 72 are less important in the reaction with sulfite oxidase than with cytochrome b_5 (Fig. 3, Table II). Our studies [8,28] on the reaction between cytochrome *c* and cytochrome b_5 support the theoretical model of Salemme in which cytochrome *c* lysines 13, 27, 72, and 79 form complementary charge pair interactions with cytochrome b_5 carboxylate groups Glu 52, Glu 48, Asp 64 and the exposed heme propionate, respectively [21]. They also indicate that lysines 8, 25, 87 and probably 86 are involved in somewhat weaker electrostatic interactions with cytochrome b_5. Dailey and Strittmatter [29] have used chemical modification techniques to show that Glu 47, Glu 48, Glu 52, and the exposed heme propionate of cytochrome b_5 are involved in electrostatic interactions with cytochrome b_5 reductase. However, none of the four acidic amino acid residues which are involved in the reactions of cytochrome b_5 are conserved in the sequence of chicken sulfite oxidase heme domain, three of the four being replaced by neutral amino acids [5—7]. There are in fact only 26 identical amino acids in the homologous sequences of chicken sulfite oxidase heme peptide and chicken cytochrome b_5. Although the amino acid sequence of beef sulfite oxidase heme peptide has not yet been reported, it is likely that there will be enough differences from that of beef cytochrome b_5 to explain the differences we have observed between the interaction domains of the two proteins with cytochrome *c*.

The lysine modification studies summarized in Table I and Fig. 3 have established that the interaction domains on cytochrome *c* for sulfite oxidase and cytochromes b_5, c_1, and oxidase are all located at the heme crevice, suggesting a common electron transfer mechanism. It appears that the highly conserved lysines surrounding the heme crevice of cytochrome *c* have evolved to direct the interaction of cytochrome *c* with its redox partners. The close correspondence between the interaction domains for sulfite oxidase and cytochrome oxidase suggests that cytochrome *c* probably undergoes some type of limited rotational or translational diffusion as it transports electrons between these two proteins.

Acknowledgement

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