periments (8-btcAMP, in particular) were similar or identical to those in which phosphorylation in rat myotubes is demonstrated (*7*). Our results would therefore imply that in contrast to nicotinic receptors from *Torpedo* (*9*), the extent of receptor phosphorylation in rat myoballs did not influence the time course of desensitization. One explanation for this difference might be that cAMP-dependent phosphorylation of the γ subunit, which occurs in *Torpedo* but not in rat myotubes, accounts for the acceleration of desensitization observed with *Torpedo* receptors. Because the proposed site of γ subunit phosphorylation by cAMP-dependent protein kinase is missing in most other species (*19*), our results may not be surprising (*20*).

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12. The concentrations of FSK used here are similar to those that accelerate desensitization at rat soleus endplates (*5*) or cause substantial phosphorylation in rat myotubes (*7*). In contrast, FSK (but not dideoxy-FSK) has also been reported to have similar desensitization effects (also at rat soleus endplates) after a 30-min exposure to concentrations ≤1 μ*M* (*4*). In myotubes, 1-hour treatments with such low concentrations of FSK do not effect appreciable phosphorylation (*7*).
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20. Our experiments were completed on 4- to 7-day-old cultured cells in which more than 99% of channels are small-conductance fetal-type [S. A. Siegelbaum, A. Trautmann, J. Koenig, *Dev. Biol.* **104**, 366 (1984)], which are associated with a subunit composition of $\alpha_2\beta\gamma\delta$ [M. Mishina *et al.*, *Nature* **321**, 406 (1986)].
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25. The intracellular (pipette) solution contained 127 m*M* sodium aspartate, 30 m*M* KCl, 1 m*M* $MgCl_2$, 2 m*M* magnesium adenosine triphosphate, 10 m*M* Hepes, and 10 m*M* glucose; *p*H 7.4; osmolality, 300 to 308 mos*M*. The extracellular solution contained 127 m*M* NaCl, 10 m*M* KCl, 500 n*M* tetrodotoxin, 10 m*M* Hepes, and 10 m*M* glucose; *p*H 7.4; osmolality 285 to 300 mos*M*. The extracellular solution also contained 0 to 5 m*M* $CaCl_2$ and 1 to 4 m*M* $MgCl_2$. In control experiments, varying the external $CaCl_2$ and $MgCl_2$ concentrations had no effect on the rate of ACh current desensitization.
26. Forskolin and 1,9-dideoxy-FSK (Calbiochem) were maintained as stock solutions in dimethylsulfoxide (DMSO). At the concentrations that resulted after final dilution (0.25% or less), the solvent had essentially no effect on the time course of desensitization ($n = 2$). Because of its lipophilicity, 1,9-dideoxy-FSK showed a marked tendency to crystallize out of DMSO upon dilution. To maintain 1,9-dideoxy-FSK in solution, heated extracellular solution was added to the stock dropwise while vortexing. L858051 (Calbiochem) was added directly to the intracellular solution (*25*).

27. We thank R. Dingledine, G. Oxford, and T. K. Harden for comments on the manuscript, and G. Scarborough for helpful discussions. Supported by NIH grant GM32211 and the University of North Carolina Research Council.

10 February 1988; accepted 14 April 1988

Probing the Mechanisms of Macromolecular Recognition: The Cytochrome b_5–Cytochrome c Complex

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The specificity of complex formation between cytochrome b_5 (cyt b_5) and cytochrome c (cyt c) is believed to involve the formation of salt linkages between specific carboxylic acid residues of cyt b_5 with lysine residues on cyt c. Site-directed mutagenesis was used to alter the specified acidic residues of cyt b_5 to the corresponding amide analogues, which resulted in a lower affinity for complex formation with cyt c. The dissociation of the complex under high pressure resulted in specific volume changes, the magnitude of which reflected the degree of solvation of the acidic residues in the proposed protein-protein interface.

ALTHOUGH MUCH EFFORT HAS BEEN expended on the mechanisms of protein–nucleic acid recognition and multisubunit interactions (*1*), considerably less insight is available into the details surrounding the establishment of specific heterologous protein-protein complexes. Perhaps the most widely studied model system is a complex between mammalian cyt b_5 and cyt c (*2–7*). Cytochrome b_5 is a 17,000-dalton heme protein involved in the transfer of reducing equivalents to a variety of physiological acceptors. In the hepatic endoplasmic reticulum, cyt b_5 serves as the electron donor to the fatty acid desaturase complex and to cytochrome P-450 (*8–14*). A similar cyt b_5 lacking a 35–amino acid membrane-anchor domain acts as a soluble reductant of hemoglobin in erythrocytes (*15, 16*). Cytochrome c serves as a carrier in the mitochondrial electron transfer system and, although it does not normally meet cyt b_5 physiologically, it can serve as a facile in vivo acceptor. Direct evidence for the formation of a stoichiometric complex between these two proteins was obtained by Mauk *et al.* (*3*) and by La Mar (*17*). Kinetic documentation of electron transfer from the heme of cyt b_5 ($E_0' = +6$ mV) to that of cyt c ($E_0' = +260$ mV) in a diprotein complex was observed by McLendon *et al.* (*4*).

With the use of a high-resolution three-dimensional structure of both cyt b_5 and cyt

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Table 1. Proposed surface charge interactions in cyt b_5 with several redox proteins at *p*H 7 and *p*H 8. Abbreviations: cyt c, horse heart cyt c; Hb, β_2 subunit of horse methemoglobin; and Myo, bovine heart myoglobin.

*p*H 7				*p*H 8	
Cyt b_5	Cyt c	Hb*	Myo†	Cyt b_5	Cyt c
E48	K13	K59		E48	K72
E44	K27	K61	K50	E13‡	K79
E43	K25	K65	K47		
D60	K72	K95	K98	D60	K87
Heme-COO-	K79	K66		E56	K86

*Proposed docking based on computer simulation (*19*). †Docking based on computer simulation and ^{1}H nuclear magnetic resonance studies (*20*). ‡In the modeling studies bovine cyt b_5 was used. All of the residues referred to above in the bovine cyt b_5 are conserved in our rat liver sequence (with the exception of Glu^{13}, which is Gln^{13} in the rat liver sequence).

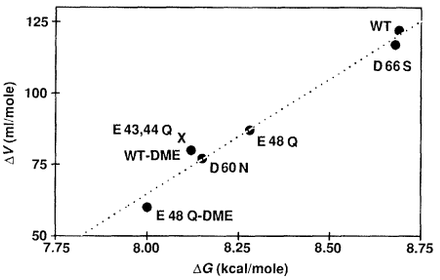

Fig. 1. Linear free energy relation for the association of cyt b_5 and cyt c; WT, wild type.

c, a model for the formation of a specific complex between these two cytochromes was initially proposed by Salemme and co-workers (*18*). In this model, the specific orientation of the heme groups of these two cytochromes was realized through the selective formation of distinctive salt bridges between the acidic residues of cyt b_5 and the positively charged lysine residues of cyt c. Similar theoretical models of specific salt bridge linkages have also been suggested by computer graphics "docking" of cyt b_5 and hemoglobin (*19*) and myoglobin (*20, 21*) (Table 1). Experimental evidence for these proposed protein-protein aggregate structures has been more difficult to obtain. Millet and co-workers (*6*), using chemical derivatization of cyt c, substantiated the structure of the Salemme model (*18*) and proposed an additional E43-K25 salt bridge (Table 1). Mauk and co-workers (*5*), using cyt b_5 reconstituted with iron–protoporphyrin IX dimethyl ester (DME), suggested a slightly different orientation of the cyt c–cyt b_5 complex when the heme-propionate salt bridge is removed. However, the complete experimental documentation of those residues of each protein partner that are involved in recognition and the formation of the specific complex architecture and protein-protein interface domain needs further documentation. We describe the results of site-directed mutagenesis of a totally synthetic rat hepatic cyt b_5 gene (*22*) that selectively alters the salt bridges thought to be involved in protein-protein recognition, and demonstrate the use of high-pressure techniques to document the role of hydrogen bonding and salt bridge formation in macromolecular recognition.

The application of high pressure to the study of biological systems leads to the determination of equilibrium and activation volume changes and can often provide insight into the mechanisms and driving forces for the establishment of protein-protein aggregates (*23*). In particular, volume changes quantitated as the variation in the dissociation constant for protein-protein complex

Table 2. Dissociation of cyt b_5 and cyt c, as obtained in tris-HCl buffer at an ionic strength of 2 m*M*, *p*H 7.4 (except where noted as *p*H 8). Errors (SEM) are 0.08 kcal/mole in ΔG and 5 ml/mole in ΔV.

Cyt b_5	K_d ($M \times 10^6$)	$-\Delta G$ (kcal/mole)	$-\Delta V$ (ml/mole)
Wild type	0.40	8.69	122
E 48 Q	0.80	8.28	87
D 60 N	1.00	8.15	77
E 43, 44 Q	1.10	8.09	85
WT-DME	1.05	8.12	80
E 48 Q–DME	1.30	8.00	60
D 66 S*	0.41	8.68	117
WT (*p*H 8)	1.47	7.92	105
WT-DME (*p*H 8)	0.22	9.04	96

*As a control, D66 was changed to S66. If this residue, which is near the proposed interfacial domain, were sequestered from solvent, one would expect a large decrease in the magnitude of ΔV, which was not observed.

formation can be directly related to the exposure of charges that are sequestered from solvation at the protein-protein interface. The application of high pressure dissociates a physiologically active complex between detergent solubilized and purified cyt b_5 and cytochrome P-450_{LM2} from hepatic microsomes (*24*). The analysis of this hepatic system, however, is complicated by aggregate formation of the dissociated amphipathic proteins. We have used high-pressure methodologies to quantitate the free energies and volume changes for the formation of a functional, soluble cyt c–cyt b_5 complex and have also used site-directed mutagenesis to alter the carboxylic acid residues of cyt b_5 that have been proposed to provide specific salt bridges across the interface domain to cyt c. The residues D60, E48, and E43 and E44 were individually replaced by their amide analogues, N60, Q48, and Q43 and Q44, respectively. The Gln and Asn residues were chosen as replacements for their corresponding acid side chains, as they are nearly isosteric and can also form weaker hydrogen bonds with the lysine residues of cyt c with similar stereochemistry, but would not display a large solvation volume change on dissociation of the complex. The heme propionate–lysine salt bridge proposed to form across the dimer interface (Table 1) was selectively removed from participation by reconstituting cyt b_5 with DME.

Direct quantitation of the formation of a functional cyt b_5–cyt c complex can be realized through optical difference spectroscopy (*3*). This same optical spectral perturbation can be used to follow the dissociation of the diprotein complex with increasing hydrostatic pressure. Table 2 lists the dissociation constants, the Gibbs free energies (ΔG), and the volume changes (ΔV) derived for cyt b_5–cyt c binding.

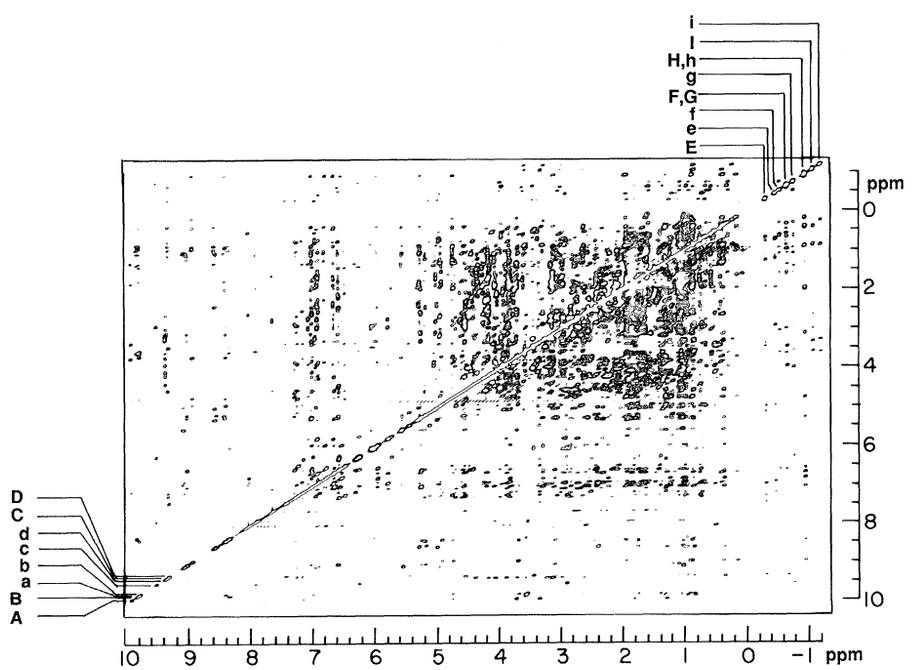

Fig. 2. Phase-sensitive 500-MHz ^{1}H NOESY spectrum of wild-type reduced cyt b_5 (5 m*M* in 0.05*M* K_iPO_4, *p*H 6.8 in D_2O, 25°C, 1024 real points in t_2, and 310 t_1 values; spectrum is unsymmetrized). Two possible heme binding orientations resulted in the appearance of doublets for heme binding site resonances. Resonances marked in capital letters resulted from one heme orientation, and those in lower case letters resulted from the other: (A, b), heme δ-meso; (B, a), β-meso; (C, d), γ-meso; (D, c), α-meso; (E, e), L25 δ_2 methyl; (F, f), L46 δ_1 methyl; (G, g), L46 δ_2 methyl; (H, h), I76 δ methyl; and (I, i), V61 γ_2 methyl. Chemical shift and NOE cross-peaks for L25, L46, and V61 methyl groups provided sensitive probes of tertiary structure in the region involved in protein-protein recognition. Based on these criteria, only E 43, 44 Q showed significant variation in tertiary structure with respect to the wild type.

The wild-type cyt b_5–cyt c complex is formed with a ΔG of −8.69 kcal/mole (298 K, 1*M*). Removal of proposed salt bridges by site-directed mutagenesis (*25, 26*) resulted in a decrease in the overall affinity of the two proteins. Since each salt bridge in the complex is differentially solvated and could potentially display different stereochemical configuration, there is no a priori reason to expect the decrease in binding free energy to be the same for each salt bridge removed. Greater insight into the role of the salt bridges in the determination of the specificity of protein-protein complex formation is realized through the study of dissociation volumes. If a salt bridge is sequestered in the protein-protein interface, the dissociation of the complex will result in an increased solvation of the exposed charges, and through electrostriction, a net decrease in system volume. Thus, through Le Chatelier's principle, high hydrostatic pressure will tend to dissociate the cyt b_5–cyt c complex.

Complete salt bridge solvation was previously estimated to result in volume change of −25 to −30 ml/mole (*27*). With an observed dissociation volume for the wild-type cyt b_5–cyt c complex of −122 ml/mole, one could expect that about four to five salt bridges accounted for the observed specificity of protein-protein interaction if all of the volume changes were due to the exposure of completely sequestered salt linkages. This interpretation is consistent with the theoretical proposals for specific ion pairs (Table 1) and with the decrease in dissociation volume for the complex by roughly 40 ml/mole for each singly mutated salt linkage.

The contribution of salt linkages to the specificity of the formation of the cyt b_5–cyt c complex can be visualized with a linear free energy relation (Fig. 1). Here an excellent linear relation is observed between the $P\Delta V$ work (*P*, pressure) and the change in total ΔG for complex formation. The different volume changes observed through the replacement of individual salt bridges through the site-directed mutagenesis experiments presented all appear to scale with the degree of solvation in the protein-protein interface (*18*), and thus a major contribution to the total volume change of the association is through these salt linkages.

Although this linear free energy relation is obeyed remarkably well, two points need to be discussed. The E 43, 44 Q double mutation leads to changes in ΔV and ΔG that do not exactly follow the simple relation in Fig. 1. A differential contribution to $P\Delta V$ and ΔG would occur if an ion pair between E43 and K25 contributed to the overall association free energy of the complex through enthalpic and entropic terms, yet not be completely isolated from solvation in the protein-protein dimer. In this case, the contribution to $P\Delta V$ due to solvent electrostriction on complex dissociation would be minimal, which indeed is suggested from the proposed docking structure of cyt c and cyt b_5 (*18*). The second point concerns the thermodyamic parameters for the double mutant E 48 Q–DME. This modified cytochrome with both E48 and heme propionate substitutions cleanly obeys the linear free energy relation, which suggests that solvation factors contribute proportionately to ΔG and $P\Delta V$. The nonadditivity of the single-mutant free energies indicates the presence of an interaction free energy in the double mutant, and suggests that either the introduction of the DME substitution has allowed solvation of the interface between cyt b_5 and cyt c or that there is significant electrostatic interaction between the E48 carboxylate and the heme propionate (*28*).

We performed a series of NOESY (two-dimensional nuclear Overhauser effect) nuclear magnetic resonance experiments (Fig. 2) to determine whether the surface charge mutations significantly effect the tertiary structure of cyt b_5. These spectra revealed no significant structural fluctuations upon mutation of surface charges except for the E 43, 44 Q double mutant, in which case some localized structural change in the helix containing the 43 and 44 residues is seen (*29*).

The cyt b_5–cyt c complex has been proposed to form an alternate complex under more basic conditions (*p*H 8) (*5*), wherein the interface domain shifts to form salt linkages with E13 and E56 rather than E44 and the heme proprionate (Table 1). The presence of Gln^{13} residue in the rat liver sequence as opposed to the Glu residue of the bovine sequence allows us to test this hypothesis with the ΔG changes and pressure titrations of wild-type and the DME-reconstituted protein (WT-DME) with cyt c. As found by Mauk *et al.* (*5*), the WT-DME protein binds tighter to cyt c than cyt b_5 does at any *p*H (Table 2). For the wild-type b_5, Mauk *et al.* suggests that at *p*H 8.0, there is a 3:2 distribution between the complex proposed by Salemme and their alternative complex structure. The decrease in volume from −122 ml/mole (*p*H 7.4) to −105 ml/mole (*p*H 8.0) that we obtained is consistent with this model, in that at *p*H 8.0 the E44 carboxylate and heme proprionate would be partially solvated, and that the volume change increased for the WT-DME from −80 ml/mole (*p*H 7.4) to −96 ml/mole (*p*H 8.0). This latter result suggests that at *p*H 8.0 the esterified heme propionate is not disrupting the formation of a salt bridge to the same extent as at *p*H 7.4.

Thus the formation of specific stereochemical salt linkages in the cyt b_5–cyt c complex plays a crucial role in maintaining the appropriate relative orientations and nuclear coordinates for the activated donor and acceptor structures to favor electron transfer.

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21. Abbreviations for amino acid residues are: D, Asp; E, Glu; I, Ile; K. Lys; L, Leu; N, Asn; Q, Gln; S, Ser; and V, Val. A mutation replacing Glu^{43} and Glu^{44} with glutamines is written as E 43, 44 Q.
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25. The techniques used for site-directed mutagenesis on the synthetic rat liver cyt b_5 gene are described in (*22*) and referenced in (*26*). To synthesize the charge mutants, the wild-type cyt b_5 synthetic gene was cut with the restriction enzymes Sma I and Xho I, and the large fragment was isolated by gel electrophoresis. New oligonucleotides were synthesized in which the acidic amino acid codon was replaced by the codon for the appropriate amide. For example, the codon for D60 (GAC) was replaced with AAC to give N60 in the mutant protein. These oligonucleotides were mixed with equal molar amounts of the remaining three wild-type oligonucleotides, heated to 95°C and slowly cooled, ligated to the Sma I–Xho I cut DNA at a 10:1 molar ratio with T4 DNA ligase, and transformed into *Escherichia coli* TB-1 (BRL Incorporated). Recombinant colonies were screened by colony hybridization and were verified by DNA sequencing.
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30. Supported through NIH grants GM 33775 and GM 31756. We gratefully acknowledge numerous discussions with G. Weber and G. La Mar, as well as T. Harrigan's aid in construction and operation of our high-pressure apparatus.

28 December 1987; accepted 22 April 1988