Description of the Molecular Mechanism of Cooperativity in Human Hemoglobin Cannot Be Limited to a First-Order Free Energy Coupling Concept†

Michael L. Johnson

Department of Pharmacology, Interdisciplinary Biophysics Program, Diabetes Research and Training Center, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Received April 8, 1987; Revised Manuscript Received September 24, 1987

ABSTRACT: Studies of the linkage between ligand binding and subunit assembly of oligomeric proteins have extensively used the concept of free energy coupling. The "order" of these free energy couplings was introduced [Weber, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7098–7102] as the number of subunits that must be liganded to alter specific intersubunit interactions. This concept dictates that the ligation of fewer subunits has no effect, but once the order number of subunits becomes ligated, the specific intersubunit interaction energy between those particular subunits is completely eliminated. Weber's report claims that the free energy coupling between oxygen binding and the dimer–tetramer subunit assembly in stripped human hemoglobin A is "first order". This conclusion is based on the analysis of a set of previously published equilibrium constants [Mills, F. C., Johnson, M. L., & Ackers, G. K. (1976) *Biochemistry 15*, 5350–5362]. I subsequently reported that the original experimental data, from which the equilibrium constants were derived, are consistent with both the first-order and "second-order" free energy coupling concepts [Johnson, M. L. (1986) *Biochemistry 25*, 791–797]. I also demonstrated that more precise recent experimental data [Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry, 23*, 604–617] are consistent with both the first-order and second-order free energy coupling concepts. A recent article [Weber, G. (1987) *Biochemistry 26*, 331–332] disagrees that the oxygen-binding data for human hemoglobin A are consistent with a second-order model. This paper explains the difference in an assumption that led to different conclusions. In my first paper (Johnson, 1986) I assumed that $\delta_{2\alpha}$ need not be equal to $\delta_{4\alpha}$ and that $\delta_{2\beta}$ need not be equal to $\delta_{4\beta}$. With that assumption I demonstrated that both the first- and second-order free energy coupling concepts are consistent with the experimental data. This paper demonstrates, assuming $\delta_{2\alpha}$ need not be equal to $\delta_{4\alpha}$ and $\delta_{2\beta}$ need not be equal to $\delta_{4\beta}$, that *both* the first- and second-order free energy coupling concepts predict a tetrameric molecule in which oxygenation promotes dissociation. If the assumption is made that $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$, then *neither* the first- nor the second-order free energy coupling concept is consistent with the most current and precise experimental data (Chu et al., 1984). Consequently, with either assumption the first-order free energy coupling concept is *not* a necessary and sufficient condition to describe the molecular mechanism of cooperativity in human hemoglobin A.

The binding of oxygen to human hemoglobin A has been extensively utilized for the analysis of "models" of cooperativity in oligomeric proteins [cf. Ackers and Johnson (1981), Herzfeld and Stanley (1974), Johnson (1986), Johnson and Ackers (1982), Johnson et al. (1984), Koshland et al. (1966), Lee and Karplus (1983), Monod et al. (1965), Perutz (1970a,b), Szabo and Karplus (1972), and Weber (1972, 1982, 1984, 1987)]. Human hemoglobin A has been utilized for this purpose because of the large amount of experimental information that is available from numerous experimental procedures [cf. Baldwin and Chothia (1979), Perutz et al. (1969), Eisenberger et al. (1978), Asher et al. (1981), Russu et al. (1983), Viggiano and Ho (1979), and Viggiano et al. (1979)]. Of particular importance to the understanding of the cooperative mechanism of oxygen binding by human hemoglobin A is a knowledge of the free energy changes that are concomitant with the oxygenation–deoxygenation cycle of human hemoglobin [cf. Ackers (1980), Ackers and Halvorson (1974), Ackers and Johnson (1981), Chu et al. (1984), Flanagan et al. (1981), Johnson and Ackers (1982), Mills and Ackers (1979), Mills et al. (1976), Pettigrew et al. (1982), and Smith and Ackers (1985)]. This large library of experimental results imposes stringent constraints on the types of models for the interactions responsible for cooperativity in human hemoglobin A.

Weber (1984) claims to have demonstrated that the concept of a first-order free energy coupling is necessary and sufficient to describe the linkage between subunit assembly and oxygen binding in human hemoglobin A. The order of free energy coupling refers to the number of human hemoglobin A subunits that must be liganded in order to induce a complete elimination of the free energy of interaction between the specific liganded subunits. It does not refer to a specific sequence of adding ligands to the human hemoglobin A tetramer.

I recently published a paper (Johnson, 1986) in which it was demonstrated that free energy couplings of both first and second order are capable of describing the oxygen-binding data which Weber (1984) indirectly used, as well as more precise recent experimental data (Chu et al., 1984). Most recently, Weber (1987) claims to have found an inconsistency in the calculations that led to my conclusions.

It is a difference in a basic assumption in the analysis that has led to these apparently contradictory conclusions. If one assumes that the intrinsic affinities of the α and β chains within the hemoglobin tetramer are equal to the intrinsic chain affinities within the hemoglobin dimer, as Weber (1984, 1987) did, then neither the first- nor second-order free energy coupling concept is consistent with all of the currently available experimental data. If the affinities are not assumed to be

† This work was supported by Grants AM-22125 and GM-28928 from the National Institutes of Health.

0006-2960/88/0427-0833$01.50/0

equal, as per my paper (Johnson, 1986), then both the first-order and second-order free energy coupling concepts are consistent with the actual experimental data. In either case, it is not experimentally justified to limit the description of the molecular mechanism of cooperativity in human hemoglobin A to a first-order free energy coupling concept.

Experimental Data Employed

Two independent sets of previously published data were used in this study (Mills et al., 1976; Chu et al., 1984). Both sets pertain to the binding of oxygen to stripped human hemoglobin A as a function of both hemoglobin concentration and oxygen concentration.

In these sets of data the binding of oxygen was measured as a function of oxygen concentration at a range of human hemoglobin A concentrations. It has been shown that at low concentrations of hemoglobin a significant fraction of the oxygenated human hemoglobin A will occur as dimers, while the deoxygenated hemoglobin will exist predominantly as tetramers (Ackers & Halvorson, 1974; Johnson & Ackers, 1977; Johnson et al., 1976; Mills et al., 1976; Valdes & Ackers, 1978). In addition to the oxygen-binding data, each of these sets of data includes an independent evaluation of the dimer to tetramer association constant for both oxygenated and deoxygenated human hemoglobin A. In Weber's description of the order of free energy coupling (1984, 1987), he used equilibrium constants that Mills, Johnson, and Ackers (1976) evaluated from the Mills et al. (1976) data set by a model-independent thermodynamic analysis.

For the analysis presented here the original oxygen-binding data were used rather than equilibrium constants derived from the data. To ensure statistical validity, the nonlinear least-squares analysis method requires using the actual experimental observations and their concomitant experimental uncertainties. This allows for a comparison of different mechanisms of oxygen binding and different relative minima of the same mechanism on the basis of changes in the variance of the nonlinear least-squares analysis.

Order of Free Energy Coupling Concept

The most general case of the first- and second-order free energy coupling concepts applied to human hemoglobin A incorporates 10 distinct parameters. Six of these describe the oxygen-binding properties of the tetrameric oligomer, three describe the oxygen-binding properties of the dimeric oligomer, and one refers to the self-association of the dimers to form tetramers.

In the free energy coupling concept of the human hemoglobin A tetramer, there are six possible oxygenation-sensitive free energies of interaction between the following pairs of subunits: $\alpha^1\beta^1$, $\alpha^2\beta^2$, $\alpha^1\beta^2$, $\alpha^2\beta^1$, $\alpha\alpha$, and $\beta\beta$ (Weber, 1984, 1987; Johnson, 1986).[1] Oxygenation-sensitive constraints are defined as those constraints that are altered by the state of oxygenation of the particular human hemoglobin A oligomer. These thermodynamic constraints, or free energies of interaction, are defined to exist if neither of the paired subunits is oxygenated. For first-order free energy coupling, these constraints are released if either of the paired subunits is oxygenated. For second-order free energy coupling, the constraints are released only when both of the subunits are oxygenated. Therefore, for these types of interactions the reference state is defined to be the fully oxygenated tetramer. In the present paper it is assumed that $\alpha^1\beta^1 = \alpha^2\beta^2$ and $\alpha^1\beta^2 = \alpha^2\beta^1$. These two types of interactions are referred to as $\alpha^i\beta^i$ and $\alpha^i\beta^j$, respectively, where i is not equal to j. A summary of the number of constraints of each of these types for both first- and second-order free energy coupling for each of the 10 possible ways to partially or fully oxygenate the human hemoglobin A tetramer can be found in Johnson (1986).

Two additional parameters are required to fully describe the binding of oxygen to tetrameric human hemoglobin A. These two parameters, $\delta_{4\alpha}$ and $\delta_{4\beta}$, are the free energies of interaction of oxygen with the individual α and β subunits in tetrameric human hemoglobin A. These composite parameters include all of the possible interactions within the human hemoglobin A tetramer that are not specifically addressed by the $\alpha^i\beta^i$, $\alpha^i\beta^j$, $\alpha\alpha$, and $\beta\beta$ described above. Most specifically, the $\delta_{4\alpha}$ and $\delta_{4\beta}$ parameters include a contribution from any oxygenation-*insensitive* constraints imposed along the $\alpha^i\beta^j$, $\alpha\alpha$, and $\beta\beta$ interfaces when the individual $\alpha\beta$ dimers associate to form the tetramers. Since these two parameters, $\delta_{4\alpha}$ and $\delta_{4\beta}$, are actually composites of a large number of different types of interactions, it is unrealistic to assume that both parameters will necessarily have the same numerical value. In light of the recent discussion in the literature (Noble, 1983; Peller, 1982; Weber, 1982, 1984) pertaining to the molecular origin of asymmetry of ligand binding as it relates to differences in the α and β subunits, it is critically important that $\delta_{4\alpha}$ and $\delta_{4\beta}$ *not be required*, or assumed, to have the same meaning or numerical value.

The mathematical formulation for the dimeric species is analogous to that for the tetrameric oligomers. Two parameters, $\delta_{2\alpha}$ and $\delta_{2\beta}$, pertain to the properties of the dimeric oligomers and are analogous to the tetramer parameters $\delta_{4\alpha}$ and $\delta_{4\beta}$. These dimer parameters are composites of a number of, but not all of, the same interactions that were summed into the corresponding tetramer parameters. It cannot be assumed a priori that $\delta_{2\alpha}$ and $\delta_{2\beta}$ have the same conceptual meaning as the respective tetramer parameters or that they have the same numerical values. Interactions of a type analogous to $\alpha^i\beta^i$ could also occur in the dimer oligomers and are referred to as $\delta_{\alpha\beta}$. The free energy coupling concept thus requires three parameters to describe the oxygen-binding properties of the dimeric oligomers, i.e., $\delta_{2\alpha}$, $\delta_{2\beta}$, and $\delta_{\alpha\beta}$. It has been shown [cf. Mills et al. (1976) and Mills and Ackers (1979)] that the dimeric oligomers bind oxygen noncooperatively. I have therefore assumed, as Weber did in his reports on the order of free energy coupling (Weber, 1984, 1987), that $\delta_{2\alpha}$ is equal to $\delta_{2\beta}$ and that no oxygenation-sensitive interactions, $\delta_{\alpha\beta}$, exist in dimeric oligomers.

The free energy coupling concept does not explicitly include a formulation of the interactions involved in the dimer to tetramer polymerization that are not sensitive to oxygenation. Consequently, a mathematical formulation of the concept requires that one of the dimer to tetramer free energies of association be included. We chose ${}^0\Delta G_2$, the free energy to form deoxygenated tetramers from deoxygenated dimers, because it has been measured independently under the same experimental conditions (Ip et al., 1976; Mills et al., 1976; Chu et al., 1984).

Mathematical Formulation

The translation of the thermodynamic concept of order of free energy coupling into a mathematical expression that

[1] The parameter names used in this manuscript, $\alpha^1\beta^1$, $\alpha^2\beta^2$, $\alpha^1\beta^2$, $\alpha^2\beta^1$, $\alpha\alpha$, and $\beta\beta$, were defined by Weber in his 1984 publication and used subsequently in the literature discussion (Johnson, 1986; Weber, 1987). An earlier work of mine (Johnson et al., 1984) used the same names for the parameters of a different mechanistic description of the functioning of human hemoglobin. Please note that the calculations present here and in my previous report on the order of free energy coupling (Johnson, 1986) are not in any way dependent on the Johnson–Turner–Ackers mechanistic model of hemoglobin (Johnson et al., 1984).

describes fractional saturation as a function of oxygen and hemoglobin concentrations requires two types of information. First, the allowed types of thermodynamic interactions between individual constituents of the molecule must be explicitly defined. These free energies of interactions are referred to as thermodynamic constraints upon the system. The second type of required information is an explicit statement of the rules by which these constraints are altered during the functional cycle. For the present example the functional cycle is the oxygenation–deoxygenation of the hemoglobin and its concomitant subunit dissociation and association. Once the constraints and rules have been defined an equation that describes the fractional saturation with oxygen as a function of oxygen concentration and protein concentration can be formulated. This equation is then utilized by a nonlinear least-squares fitting algorithm to estimate the numerical values of the free energy coupling parameters with the highest probability of being correct, the confidence intervals for the estimated parameters, and various measures of the goodness-of-fit such as the variance.

Once the constraints and rules have been defined, the formalisms of statistical thermodynamics are employed to translate the thermodynamic concepts into equations describing the oxygen-binding data. The statistical thermodynamic approach utilizes partition functions (Herzfeld & Stanley, 1974; Hill, 1960; Johnson, 1986; Johnson & Ackers, 1977; Johnson et al., 1984; Lee & Karplus, 1983; Noble, 1983; Peller, 1982; Szabo & Karplus, 1972), which are sometimes called binding polynomials. Derivations of the statistical thermodynamic equations are presented elsewhere (Johnson, 1986) and will not be repeated here.

The nonlinear least-squares procedure that was used for the estimation of these parameters from the actual experimental observations has been presented elsewhere (Atha et al., 1979; Johnson, 1983; Johnson & Ackers, 1977, 1982; Johnson et al., 1981; Johnson & Frasier, 1985; Johnson et al., 1976) and will not be restated here.

For a complete definition of the thermodynamic parameters, the reader is referred to the literature (Ackers, 1980; Ackers & Halvorson, 1974; Ackers & Johnson, 1981; Atha et al., 1979; Chu & Ackers, 1981; Chu et al., 1984; Flanagan et al., 1981; Ip et al., 1976; Johnson, 1986; Johnson & Ackers, 1977, 1982; Johnson et al., 1976, 1984; Mills & Ackers, 1979; Mills et al., 1976; Pettigrew et al., 1982; Smith & Ackers, 1983; Valdes & Ackers, 1978).

Results and Discussion

There is one significant difference in an assumption used in my previous paper on the order of free energy couplings (Johnson, 1986) and Weber's recent publication (Weber, 1987). I assumed that the intrinsic affinities of the hemoglobin chains within tetrameric human hemoglobin A_0, $\delta_{4\alpha}$, and $\delta_{4\beta}$, are not required to be the same as the intrinsic affinities of the hemoglobin chains within dimeric hemoglobin, $\delta_{2\alpha}$ and $\delta_{2\beta}$. Conversely, Weber has assumed that $\delta_{4\alpha} = \delta_{2\alpha}$ and $\delta_{4\beta} = \delta_{2\beta}$. Weber did note this difference in his recent paper (Weber, 1987), but he misinterpreted its significance, thus obtaining conflicting results from the second-order coupling analysis that I described in the previous work (Johnson, 1986).

In Weber's analysis (Weber, 1987) of the second-order free energy coupling concept, he compared the total free energy to oxygenate two isolated α and two isolated β chains [$dG°$(sub) by his notation] with the free energy to totally oxygenate the tetramer of human hemoglobin A [$dG°$(tet) by his notation]. This calculation was performed on the first two columns of my previous paper's Table III (Johnson, 1986). Weber's values of $dG°$(tet) are −27.31 for the analysis based on the first-order free energy coupling concept and −27.30 for the analysis based on the second-order coupling concept. His calculation of $dG°$(sub) was performed by "adding twice the values given for the binding by the α and β chains ($\delta_{4\alpha}$ and $\delta_{4\beta}$ of the [mentioned] table)". In this context he mistook these values to be equal to the values for the isolated chains. The correct method to calculate $dG°$(sub) is to sum all of the interactions that can occur within the *dimer*, in this case twice the sum of $\delta_{2\alpha}$ and $\delta_{2\beta}$. My earlier paper states, "For these analyses the values of ${}^0\Delta G_2$, $\delta_{2\alpha}$, and $\delta_{2\beta}$ were taken to be the values that gave the lowest variance from the thermodynamic analysis in terms of the Adair constants" [see Table II of Johnson (1986)]. The values for the intrinsic affinities of dimeric human hemoglobin A that were presented in Table II of my previous publication (Johnson, 1986) are $\Delta G_{21}' = \Delta G_{22}' = -8.38$ kcal/mol. I also should have noted, to avoid misunderstanding, that for a noncooperative dimer $\Delta G_{21}'$, $\Delta G_{22}'$, $\delta_{2\alpha}$, and $\delta_{2\beta}$ are all equal by definition. The calculation of $dG°$(sub) that utilizes the correct values of the intrinsic affinities of the isolated chains, $\delta_{2\alpha}$ and $\delta_{2\beta}$, yields the value −33.52 for the analysis of both first- and second-order free energy coupling concepts.

The values of the difference, $dG°$(sub) − $dG°$(tet), i.e., −6.21 for analysis based on the first-order coupling concept and −6.22 for the analysis based on the second-order coupling concept, are identical within reasonable precision. This indicates that *both* the first- and second-order free energy coupling concepts predict a tetrameric molecule which dissociates upon oxygenation. The negligible difference in these answers leads one to conclude that both the first- and second-order free energy coupling concepts equally describe the experimental data.

My previous paper (Johnson, 1986) also indicates that *both* the first- and second-order free energy coupling concepts predict a tetrameric molecule which dissociates upon oxygenation. The values of the variance-of-fit, presented in Tables III and IV of that paper (Johnson, 1986), are nearly identical for all of the fits to a particular data set. These variances-of-fit are also virtually identical with the variances obtained from the model-independent thermodynamic analysis of the same comprehensive data sets. These data sets include a series of oxygen-binding curves at different hemoglobin concentrations as a measure of the dissociation properties of human hemoglobin A. If the first-order free energy coupling concept correctly predicts that the hemoglobin tetramer dissociates upon oxygenation and the second-order free energy coupling concept incorrectly predicts, as Weber concluded (1987), that the hemoglobin tetramer associates upon oxygenation, then the two concepts could not yield the same variances-of-fit.

Weber (1984, 1987) has assumed that the intrinsic chain affinities within the dimer and within the tetramer are equal, i.e., $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$. However, this assumption is inconsistent with the results of the model-independent thermodynamic analysis. It is experimentally observed that the fourth oxygen binds to the tetrameric oligomer with a higher affinity than the oxygens which bind to the dimeric oligomer. This phenomenon, called *quaternary enhancement*, is evident in the model-independent thermodynamic parameters (Mills et al., 1976; Mills & Ackers, 1979; Chu et al., 1984).

However, because a difference in an assumption exists between my paper (Johnson, 1986) and the papers of Weber (1984, 1987), I have repeated my calculations for the first-order free energy coupling concept, incorporating the assumption that the intrinsic chain affinities within the dimer and within the tetramer are equal, $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$. For

Table I: Free Energies (kcal/mol) of the First-Order Constraints

	Mills et al. (1976)	Chu et al. (1984)
${}^0\Delta G_2$	−14.38[a]	−14.42[a]
$\delta_{2\alpha}$	−8.38[a]	−8.32[a]
$\delta_{2\beta}$	−8.38[a]	−8.32[a]
$\delta_{4\alpha}$	−8.38[a]	−8.32[a]
$\delta_{4\beta}$	−8.38[a]	−8.32[a]
$\alpha\alpha$	−1.59 (−1.81, −1.37)[b]	−1.62 (−1.81, −1.40)[b]
$\beta\beta$	0.0[a]	0.0[a]
$\alpha^i\beta^i$	0.0[a]	0.0[a]
$\alpha^i\beta^j$	−2.29 (−2.42, −2.18)	−2.38 (−2.50, −2.27)
σ^2[c]	1.20×10^{-3}	7.18×10^{-4}
derived parameters		
$\Delta G_{41}'$	−5.71	−5.57
$\Delta G_{42}'$	−6.61	−6.52
$\Delta G_{43}'$	−6.63	−6.50
$\Delta G_{44}'$	−8.38	−8.32
${}^4\Delta G_2$	−8.19	−8.04

[a] Assumed to be equal to the values determined from the model-independent thermodynamic analysis. [b] The numbers in parentheses correspond to a confidence interval of 1 standard deviation. These are presented only for the parameters that were actual fitting parameters. [c] The lowest variance-of-fit for the model-independent thermodynamic analysis for the Mills et al. (1976) data was $\sigma^2 = 2.5 \times 10^{-5}$ and for the Chu et al. (1984) data was $\sigma^2 = 1.24 \times 10^{-5}$.

these calculations the values of $\beta\beta$ and $\alpha^i\beta^i$ were assumed to be zero as in the previous works (Weber, 1984, 1987; Johnson, 1986). ${}^0\Delta G_2$, $\delta_{2\alpha}$, and $\delta_{2\beta}$ were assumed to have the values determined by the model-independent thermodynamic analysis, as in the previous papers (Weber, 1984, 1887; Johnson, 1986).

The results of this analysis using the Mills et al. (1976) data are listed in the second column of Table I. The value used for $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$ was the one determined from the model-independent thermodynamic analysis, −8.38 kcal/mol (Mills et al., 1976). The variance-of-fit, σ^2, for the first-order free energy coupling concept is 49 times larger than the corresponding model-independent thermodynamic analysis for the same data. The number of degrees of freedom from this data set is 234. Consequently, a variance ratio of 49 corresponds to a vanishingly small probability that is a reasonable fit of the data. Therefore, the first-order free energy coupling model is inconsistent with the Mills et al. (1976) data when the assumption is made that $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$.

Table I also presents the same calculations for the more precise Chu et al. (1984) data assuming that $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta} = -8.32$ kcal/mol. The analysis of the first-order free energy coupling concept for this set of data also yields a vanishingly small probability of being a reasonable fit of the data. Consequently, the first-order free energy coupling concept is inconsistent with the Chu et al. (1986) data when the assumption is made that $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$.

In my first paper (Johnson, 1986) on the order of free energy coupling, I assumed that $\delta_{2\alpha}$ need not be equal to $\delta_{4\alpha}$ and that $\delta_{2\beta}$ need not be equal to $\delta_{4\beta}$. When that assumption was made, I demonstrated that both the first- and second-order free energy coupling concepts are consistent with the actual experimental data. In this report I have reanalyzed the data assuming that $\delta_{2\alpha} = \delta_{2\beta} = \delta_{4\alpha} = \delta_{4\beta}$ and have shown that the first-order free energy coupling concept is not consistent with the more precise Chu et al. (1984) data or the Mills et al. (1976) data. In either case the first-order free energy coupling concept of cooperativity is not a necessary and sufficient condition to describe the actual experimental data pertaining to human hemoglobin A.

References

Ackers, G. K. (1980) *Biophys. J. 32*, 331–346.

Ackers, G. K., & Halvorson, H. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4312–4316.

Ackers, G. K., & Johnson, M. L. (1981) *J. Mol. Biol. 147*, 559–582.

Asher, S. A., Adams, M. L., & Schuster, T. M. (1981) *Biochemistry 20*, 3339–3346.

Atha, D. H., Johnson, M. L., & Riggs, A. F. (1979) *J. Biol. Chem. 254*, 12390–12398.

Baldwin, J., & Chothia, C. (1979) *J. Mol. Biol. 129*, 175–220.

Chu, A. H., & Ackers, G. K. (1981) *J. Biol. Chem. 256*, 1199–1205.

Chu, A. H., Turner, B. W., & Ackers, G. K. (1984) *Biochemistry 23*, 604–617.

Eisenberger, P., Shulman, R. G., Kincaid, B. M., Brown, G. S., & Ogawa, S. (1978) *Nature (London) 274*, 30–34.

Flanagan, M. A., Ackers, G. K., Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1981) *Biochemistry 20*, 7439–7449.

Herzfeld, J., & Stanley, E. H. (1974) *J. Mol. Biol. 82*, 231–265.

Hill, T. L. (1960) *Introduction to Statistical Thermodynamics*, Addison-Wesley, Reading, MA.

Ip, S. H. C., Johnson, M. L., & Ackers, G. K. (1976) *Biochemistry 15*, 654–660.

Johnson, M. L. (1983) *Biophys. J. 44*, 101–106.

Johnson, M. L. (1986) *Biochemistry 25*, 791–797.

Johnson, M. L., & Ackers, G. K. (1977) *Biophys. Chem. 7*, 77–80.

Johnson, M. L., & Ackers, G. K. (1982) *Biochemistry 21*, 201–211.

Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301–342.

Johnson, M. L., Halvorson, H. R., & Ackers, G. K. (1976) *Biochemistry 15*, 5363–5371.

Johnson, M. L., Correia, J. J., Yphantis, D. A., & Halvorson, H. R. (1981) *Biophys. J. 36*, 575–588.

Johnson, M. L., Turner, B. W., & Ackers, G. K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1093–1097.

Koshland, D. E., Nemethy, G., & Filmer, D. (1966) *Biochemistry 5*, 365–385.

Lee, A. W., & Karplus, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 7055–7059.

Mills, F. C., & Ackers, G. K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 273–277.

Mills, F. C., Johnson, M. L., & Ackers, G. K. (1976) *Biochemistry 15*, 5350–5362.

Monod J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.

Noble, R. W. (1983) *Nature (London) 304*, 190.

Olson, J. S., Andersen, M. E., & Gibson, Q. H. (1971) *J. Mol. Biol. 246*, 5919–5923.

Peller, L. (1982) *Nature (London) 300*, 661–662.

Perutz, M. F. (1970a) *Nature (London) 228*, 726–734.

Perutz, M. F. (1970b) *Nature (London) 228*, 734–739.

Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., & Kilmartin, J. V. (1969) *Nature (London) 222*, 1240–1243.

Pettigrew, D. W., Romeo, P. H., Tsapis, A., Thillet, J., Smith, M. L., Turner, B. W., & Ackers, G. K. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1849–1853.

Russu, I. N., Ho, N. T., & Ho, C. (1983) *Biochemistry 22*, 5031–5043.

Smith, F. R., & Ackers, G. K. (1983) *Biophys. J. 41*, 415a.

Smith, F. R., & Ackers, G. K. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5347–5351.
Szabo, A., & Karplus, M. F. (1972) *J. Mol. Biol. 72*, 163–197.
Valdes, R., & Ackers, G. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 311–314.
Viggiano, G., & Ho, C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3673–3677.
Viggiano, G., Ho, N. T., & Ho, C. (1979) *Biochemistry 18*, 5238–5247.
Weber, G. (1972) *Biochemistry 11*, 864–878.
Weber, G. (1982) *Nature (London) 300*, 603–607.
Weber, G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7098–7102.
Weber, G. (1987) *Biochemistry 26*, 331–332.

Nucleotide Sequence of the *luxR* and *luxI* Genes and Structure of the Primary Regulatory Region of the *lux* Regulon of *Vibrio fischeri* ATCC 7744†

Jerry H. Devine, Cari Countryman, and Thomas O. Baldwin*
Department of Biochemistry and Biophysics, Texas A&M University, and The Texas Agricultural Experiment Station, College Station, Texas 77843

Received June 30, 1987; Revised Manuscript Received September 25, 1987

ABSTRACT: The regulation of bioluminescence in *Vibrio fischeri* involves both an autoregulatory mechanism and the adenosine cyclic 3′,5′-phosphate/*crp* system. The *lux* regulon from *V. fischeri* strain MJ-1, consisting of two operons, L (left) and R (right), and at least seven genes, *luxR* (L operon) and *luxICDABE* (R operon) and the intervening region, functions in laboratory strains of *Escherichia coli* [Engebrecht, J., Nealson, K., & Silverman, M. (1983) *Cell (Cambridge, Mass.) 32*, 773–781]. The regulatory region, consisting of *luxR*, encoding the regulatory protein, and *luxI*, encoding a function required for synthesis of the autoinducer, and the intervening region of *V. fischeri* strain ATCC 7744 has been cloned and the nucleotide sequence determined. The regulatory protein is an M_r 28 518 polypeptide consisting of 250 amino acid residues; the I protein is an M_r 21 937 polypeptide consisting of 193 amino acid residues. The *luxR* gene, the only known gene of the L operon, is transcribed in the opposite direction to the direction of transcription of the other genes of the *lux* regulon. There are 218 base pairs that separate the 5′ end of the open reading frame of the *luxR* gene from the 5′ end of the open reading frame of the *luxI* gene, the first gene in the rightward operon. In this region, there are both an apparent catabolite repressor protein binding site and an inverted repeat structure that may serve as protein binding sites for the regulation of bioluminescence.

Regulation of bioluminescence in marine bacteria has been a subject of inquiry for many years (Harvey, 1952). The first semiquantitative description of this phenomenon was presented in 1968 by Kempner and Hanson, who ascribed the lag in appearance of luminescence following inoculation of a broth culture to metabolism of inhibitors in the medium (Kempner & Hanson, 1968). Nealson et al. showed that the lag in appearance of luminescence was not due exclusively to inhibitors of the luminescence system but that the bacteria produce and secrete into the medium a compound responsible for induction of bioluminescence, and described this phenomenon as "autoinduction" (Nealson et al., 1970; Rosson & Nealson, 1981). A more detailed physiological and biochemical description of the process of autoinduction led to the isolation and structural elucidation of the autoinducer of *Vibrio fischeri* (Eberhard et al., 1981). This substance, *N*-(3-oxohexanoyl)homoserine lactone, has been synthesized and shown to function in a biological assay system (Eberhard et al., 1981; Kaplan et al., 1985). Investigation of the effect of synthetic autoinducer on expression of luminescence from a natural isolate of *V. fischeri* deficient in autoinducer synthesis confirmed that the autoinducer is both freely diffusable and effective at very low concentrations (Kaplan & Greenberg, 1985).

Following the initial cloning of *luxA* and *luxB* encoding the α and β subunits of luciferase from *Vibrio harveyi* B392 (Baldwin et al., 1984; Cohn et al., 1983), a *Sal*I fragment from *Vibrio fischeri* strain MJ-1 was cloned and shown to express the entire regulon, including genes necessary for the autoinduction phenomenon, in *Escherichia coli* strain ED8654 (Engebrecht et al., 1983). By transposon insertion mutagenesis and polypeptide synthesis in minicells, seven genes in two operons of the *lux* regulon were defined (Engebrecht & Silverman, 1984, 1986). Their organization is shown in Figure 1. The rightward operon contains *luxA* and *luxB*, which encode the α and β subunits of luciferase, as well as *luxC*, *luxD*, and *luxE*, which encode proteins required for synthesis of the aldehyde substrate, and also *luxI*, which encodes a function required for synthesis of the autoinducer molecule. The only known gene in the leftward operon, *luxR*, encodes a regulatory protein.

Earlier investigations showed that catabolite repression played a role in the regulation of bioluminescence (Nealson et al., 1970, 1972; Friedrich & Greenberg, 1983). Dunlap and Greenberg (1985), using a series of *crp* and *cya* mutants in *E. coli*, have supplied compelling evidence that the adenosine cyclic 3′,5′-phosphate (cAMP)[1]/CRP complex is indeed im-

†This research was supported by grants from the National Science Foundation (NSF DMB 85-10784) and the Robert A. Welch Foundation (A865). The generous support of the State of Texas through the Texas Advanced Technology Research Program is gratefully acknowledged.
* Address correspondence to this author at the Department of Biochemistry and Biophysics, Texas A&M University.

[1] Abbreviations: CRP, catabolite repressor protein or cyclic AMP binding protein (CAP); cAMP, adenosine cyclic 3′,5′-phosphate; bp, base pair(s); SDS, sodium dodecyl sulfate.

0006-2960/88/0427-0837$01.50/0