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DISCUSSION

Session Chairman: Alan N. Schechter *Scribe*: Preston Hensley

SCHECHTER: We have an extended comment from Allen Minton.

MINTON: An alternative estimate of the energetic contribution of oxygen-linked proton ionization to the free energy of cooperative interaction between subunits may be provided as follows: Let us define a quantity called the free energy of cooperative intersubunit interaction associated with the *i*th oxygenation step as equal to the difference between the standard state free energy of oxygenation for that step (corrected for the change in number of binding sites) and the standard state free energy of oxygenation for the fourth and final step.

$$\Delta G_i^{CI} \equiv \Delta G_i^{\circ} - \Delta G_4^{\circ}$$

The total free energy of cooperative oxygen-linked intersubunit interaction is then defined as

$$\Delta G^{CI} \equiv \sum_{i=1}^{4} \Delta G_i^{CI} = \sum_{i=1}^{4} \Delta G_i^{\circ} - 4\Delta G_4^{\circ}$$

From the linkage scheme presented by Ackers, the value of ΔG^{CI} may be calculated to be 10.16 kcal/mol or ~17 RT.

Using the method of Saroff and Minton (1972, *Science* **175**:1253), a simple expression may be derived for the contribution of oxygen-linked proton ionization to the total free energy of cooperative oxygen-linked intersubunit interaction:

$$\Delta G^{CI(H)} = -RT \ln \frac{\Pi_i (1 + k_i^{\text{oxy}}[\text{H}])^{n_i^{\text{oxy}}}}{\Pi_i (1 + k_i^{\text{deoxy}}[\text{H}])^{n_i^{\text{deoxy}}}}$$

where n_i^{oxy} and n_e^{deoxy} are respectively the numbers of oxygen-linked proton binding sites of class i in oxyhemoglobin and deoxyhemoglobin, and k_i^{oxy} and k_i^{deoxy} are respectively the apparent microscopic association constants of the oxygen-linked proton binding sites of class i in oxyhemoglobin and deoxyhemoglobin. These apparent constants will generally be functions of anion concentration (Saroff, 1972).

Using the above equation together with proton binding constants for deoxy and oxyhemoglobin derived from the differential titration data of Rossi-Bernardi and Roughton (1967, *J. Biol. Chem.* **242**:784), obtained under conditions similar to those employed by Ackers and coworkers, we calculate that $\Delta G^{CI(H)}$ is between 4 and 5 RT at pH 7.4. Thus we would estimate that 25–30% of the total free energy of cooperative interaction in oxygen binding may be attributed to oxygen-linked proton ionization under the conditions of the experiments of Ackers and collaborators.

ACKERS: This calculation tends to reinforce the idea in our paper that the energetics of proton release *per se* may contribute significantly to the free energy of cooperativity in the system. I attempted to resolve the interaction energy into enthalpic and entropic components. The significant contribution, mostly arising from the enthalpic terms. Your calculation depends on some assumptions regarding the effective pKs of the groups and the calculation is not totally model independent. It is difficult to make an accurate calculation, but the general picture that emerges from both approaches is the same.

EATON: Most biochemists who don't work on hemoglobin, and many who do, are under the impression that the two-state allosteric model provides an excellent first-order thermodynamic description of cooperative oxygen binding by hemoglobin. Since you don't mention either this work or the more detailed version of that model formulated by Szabo and Karplus, I wonder why you've apparently discarded them as a useful way of analyzing the detailed thermodynamic data.

ACKERS: I discarded them only because of the limits on length of the article, not because I thought they were unimportant. I hoped to discuss them here, in fact.

The MWC model does provide a good zeroth order approximation to many effects, including some of the reactions that I've described. Its main virtue is that it embodies in simplest form the idea that there are at least two major quaternary structure forms of the molecule. It provides a facile description but buries a lot of information. In fact it predicts insensitivity to the direct tests by which it might be excluded. By that I mean that in the two-state MWC model you have a situation where there is only one part in 10^4 or 10^5 of R-state molecules in the deoxy tetramer, making it very difficult to use any physical technique to find those molecules. If you look at the oxygenated form of the molecule, the equilibrium lies in the opposite direction and you have only 1 mol of 10^4 to 10^5 molecules in the T-state, making it virtually impossible to find those molecules. At the intermediate states of ligation one has only very small populations of molecules because of statistical factors and because of the cooperativity of the intermediate species. With singly-, doubly-, and triply-liganded species, in terms of physical detection of the quaternary conformation one must resolve fractions which might be in the neighborhood of 50/50, but the population comprising their sum is only a few percent of the total. Thus one can fit experimental data to this model, which as a first approximation is saying that the system is really described by the fully liganded and unliganded states.

We've analyzed a number of models which are more elaborate and contain more information, including the Szabo-Karplus model. The latter is particularly nice because it translates structural information, in a detailed way, relating the interactions between and within the subunit parts of the molecule. This model contains, instead of two states, 512 states of which 190 are distinguishable. There's also a model by Hertzfeld and Stanley that has 512 species for hemoglobin and other systems.

You might think that these models have exactly the opposite limitation to that mentioned above for the MWC model, but this is not true because, although models of that type contain a large number of states, the states are constrained by the rules connecting the transitions between them so you don't have as many degrees of freedom as you might expect. We have analyzed the data for the linked oxygen binding and subunit assembly in terms of more elaborate models, like the Szabo and Karplus model, which we extend with some assumptions for properties of dimers. The experimental data have now gotten us to the point where not all of the properties of the system can be explained adequately by a model even of that degree of complexity. We've also looked at quite a number of other models, involving different assumptions starting with the simplest considerations of pairwise interactions between the subunits and proceeding to more and more elaborate models. It turns out that quite a number of these models can be unequivocally excluded by the experimental data. This is an advance in the sense that for the first time in hemoglobin, at least, not all of the models can explain all of the data.

EATON: If I understand what you've said, you haven't ruled out a two-state model. You just find that it's not particularly useful when it comes to analyzing the detailed data. Is that right?

ACKERS: That's not exactly what I meant. In analyzing the linked dimer–tetramer oxygenation system according to a simple extension of the MWC model, in which we allow only two states for the entire system and assume that the

dimers have the properties of R-state tetramers, the model is unequivocally excluded by the data. So you cannot describe the complete linked system by a two-state model. But, if you allow an additional state, and assign the third state either to the tetramers (in which case the dimers have one of the three tetramer states) or to the dimers, then you can fit the data with a three-state model. In that case you can fit the tetramer with a two-state model and the dimer with an additional state.

EATON: There is one question, then, that would be particularly interesting for people who are looking at the two quarternary structures of hemoglobin and searching for a structural origin for the differences in affinity between the T- and R-states. Can you tell us whether the difference in the oxygen binding to T- and R is mainly enthalpic or mainly entropic?

ACKERS: The best estimate we can make within the context of that model is that the main difference is entropic, after we extract the contributions from the Bohr protons.

LUMRY: Gary's using a lot of words to evade the issue. The real problem in hemoglobin is one that's been a problem in water chemistry for many years. That is, you have to explain why both two-state and multistate models work. Even when very good isosbestic tests are statisfied, still one cannot confirm a two-state model. Both the two-state and the multistate models fit under one condition or another, yet only the multistate model can be correct.

ACKERS: There are some multistate models which don't fit. The general argument that you can fit all models is not correct in the case of thermodynamic information of the type I've been describing.

One can look at topological maps of pairwise interactions between the subunits, and ask whether the α-chain talks to the β-chain energetically during the course of transitions which accompany ligation. For each model we adopt a set of rules. The simplest ones assume that the energetic constraints are independent, so that when you bind an oxygen to one subunit the constraints connecting it to its neighbors are eliminated. A second class of rules assumes that the constraints are not eliminated upon ligation but that they are altered in their values. We ask the question, are there any values for these energetic constraint terms which permit one to predict experimental data using standard statistical thermodynamic methods? The answer in the case of a model which was first proposed by Linus Pauling and Jeffries Wyman is that the model doesn't fit the data at all. If we take the Perutzian topological map of salt bridges connecting the subunits with the simplest set of rules, that model doesn't fit either. If we use the second class of rules with the square topology of constraints between the subunits, which is equivalent to the Koshland-type assumption (Koshland's square model), it doesn't fit these data either. If we hybridize the Perutz and Koshland models, and use the topological map of Perutzian salt bridges with the Koshland-type assumptions, this model also doesn't fit the data. The Szabo-Karplus model, which is much more elaborate and contains more specific information, can fit the data except for the Bohr effect. If you make it fit the Bohr effect then it won't fit other properties.

There has been a general folklore in the hemoglobin world that one can fit all data to all models, particularly thermodynamic data, and that therefore thermodynamic data are useless for analyzing mechanisms or for testing possibilities. But this limitation is not intrinsic to thermodynamics nor to the hemoglobin system. It has really been a consequence of our inability to get an extensive set of self-consistent thermodynamic information providing enough degrees of constraint to test some of these models. The results of our analyses indicate that one can in fact rule out some of these models.

BLANK: The previous questioners represented biochemistry. I represent a much smaller constituency, surface chemistry. Long before molecular biologists came on the scene, scientists used to talk about macromolecules from the point of view of colloids. Surface energy was the important factor in determining what happens with a colloidal particle, e.g. whether it will disperse, whether it is stable, and so on. If one considers hemoglobin as a colloidal particle and calculates the surface energy for various processes, it is possible to show tetramer dissociation effects, the general trends of the Bohr effect, both acid and alkaline, and the dependence on ionic strength. These processes are linked through the surface energy. The Hill coefficient, a measure of cooperativity, turns out to have a simple meaning. (It is identical to the Gibbs surface excess.)

I mention this approach because when these processes are viewed in terms of surface energy, many of them are easily understood in terms of non-specific effects, and many of the special considerations that have been part of the hemoglobin field, e.g. heme–heme interactions which have been looked for and not found, are no longer needed.

ACKERS: It's nice to know that the Hill coefficient has such a simple interpretation in the case of surface modulations since it doesn't in terms of the standard equilibrium binding reactions. It is interesting how well the effects of surface area and charge density can predict the qualitative effects observed in systems of this kind.

There are two kinds of models. First, there are those which are aimed at predicting qualitatively the general behavior of systems and predicting orders of effects . Many of the papers at this symposium deal with theories of that kind. The second class of models is aimed at quantitatively accounting for the observed effects in a precise way. These

are the more difficult to develop because of the quality of the experimental information required. Also its not entirely clear once you get them to fit the data that it means anything. If you find they cannot fit the data then you learn something.

That several models of either kind can be consistent with the same data, even though based on a variety of assumptions, is really not surprising and doesn't provide very much information in my view. Progress is made by excluding models. So if you told me that surface and charge effects could not explain any of the properties of hemoglobin, that would be progress.

On the other hand what about the surfaces between the subunits that I've been talking about? To what extent are we measuring properties of interactions at the surface which might be comparable in a general way to the surface effects you're talking about and to what extent are there thermodynamic quantities actually measuring internal interactions, conformational energies and so forth? It's not possible to define rigorously the partitioning between these effects, but we can make some rough estimates which indicate that we probably are measuring interactions between the surfaces or at the surface of the molecule which might account for roughly 3/4 of the energetic effects we're looking at. Viewed this way, charge and surface area effects really probably do play an important role in these mechanisms.

BLANK: If we applied the Popper criterion, i.e. designing experiments that will negate theories rather than affirm them, I think we wouldn't have very many papers at this symposium.

One of the reasons for bringing my ideas to the attention of this audience is that I'm looking for systems in which this simple approach can be tested. I was very pleased to learn from Roland Siezen that there is evidence for an association of hemoglobin tetramers into octamers (Nichol, Siezen and Winzor, 1979, *Biophys. Chem.* **10**:7–26). This is relatively easy to understand in terms of surface energy. But it certainly doesn't seem to fit any of the other approaches discussed up to now. In other words, you can find equilibration at all levels of association if you're trying to minimize surface free energy. If you're dealing with T-states, R-states, and certain special interactions between interfaces on α-chains and β-chains, you're a little more hard pressed to come up with a consistent answer.

SCHECHTER: One of the referees, in a very long analysis, made a point which I think is worth repeating. He wondered whether or not in distinguishing salt bridges, hydrogen bonds, and Bohr protons, you are really not distinguishing between things which are very closely related, and yet getting energetics for the processes assumes that they are fundamentally different. He believes that they are just somewhat different ways of looking at the same physical processes.

ACKERS: It is difficult to make definitive statements about the pattern of energetic effects. There still aren't very good models for the energetics of pairwise interactions that would be relevant to those within a protein. One must resort to the thermochemistry of small molecules, and that's what I have done in this analysis. Although there are certainly exceptions, it seems clear that in most of the cases that have been considered and for which there is some evidence, a salt bridge involves both a hydrogen bond and an ion pair in a solvent similar to water. These salt bridges in hemoglobin are either at the surface or in clefts which are accessible to water and the energetics will be dominated by ion-pair formation such that the decrease in the electrostricted water gives a positive change in the entropy. For a hydrogen bond with no ion pair the entropy change is very likely to be negative. Ross and Subramanian have carried out an extended analysis of available information on proteins with regard to their entropies, enthalpies and heat capacities. They find conclusions very similar to mine by considering the ways in which the thermochemistry of small molecules can be compared to these processes.