KINETIC STUDIES ON RESPIRATORY PROTEINS

by

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PREFACE

The main thrust of the research that has gone into this thesis has been the study of the rapid reactions of hemoglobins and myoglobins with ligands; the reactions of hemoglobin have been under investigation for almost one hundred years, and the field remains one of the most actively investigated in molecular biology. The reasons for the intense interest are due to the fact that hemoglobin and myoglobin are two of the most important and abundant proteins in vertebrates. In the vertebrate blood stream, there are about 5 billion erythrocytes per milliliter, each erythrocyte containing about 280 million molecules of hemoglobin (1). Myoglobin is contained in muscle tissue, and is responsible for the red color of such tissues. One of the functions of hemoglobin is to bind oxygen at the lungs and carry it to the muscles, where the oxygen is transferred to myoglobin; the latter stores the oxygen until needed for metabolic oxidation. Hemoglobin and myoglobin can be studied as models for protein systems showing a specific reactivity of direct biological significance; this, coupled with the elucidation of the three-dimensional structure of the two proteins by X-ray crystallography should allow the interpretation of several physical, chemical, and functional properties of two major protein systems in structural terms.

There are many factors that facilitate research on

hemoglobins and myoglobins: the proteins are widely available, both commercially, as in the case of sperm whale myoglobin, and privately, since an immediate source of hemoglobin is no further than one's trusty right (or left) arm. The large molar absorptivity associated with the various liganded forms allows studies to be carried out at micromolar concentrations as well as allowing direct observation of reactions taking place at the active site of the protein in contrast to monitoring the appearance of product or disappearance of reactant. Both forward and reverse reactions of ligand binding, as well as equilibria, can be measured, and each reaction usually can be studied in more than one way,providing independent checks on the rate constant involved.

When studying proteins, usually great effort is expended in insuring that the protein studied is highly purified. For example, crystallization or gel filtration may be undertaken in an attempt to free the protein of interest from contamination by other protein systems. There are times, however, when the results of experiments on the molecular and catalytic properties of proteins, as well as their regulatory characteristics, can not be accounted for by assuming a homogeneous population of molecules of the protein under investigation. In kinetic studies, when heterogeneous reactions are encountered, the problem is often one of distinguishing heterogeneous subunits from heterogeneous aggregates. For two or more one-site systems,

the question that arises is whether the protein population is heterogenous or whether the reaction studied is more complex than assumed. In another case, when a protein population may consist of two or more components, one of which constitutes 90% of the population, homogeneity of reaction can be obtained within experimental error, but erroneous conclusions deduced about the system as a whole. Heterogeneity, therefore, can pose a legitimate concern.

Most proteins demonstrate heterogeneity. Enzymes that exist in more than one structural form in the same species are called isozymes (2). These are of great interest on chemical, physiological, and genetic grounds. For instance, glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle can be resolved into one minor and four major peaks of activity. Since the protein is tetrameric, this heterogeneity was attributed to the random formation of tetrameric and dimeric molecules from two similar but nonidentical subunits (3). Mitochondrial malate dehydrogenases have been shown to exist in several enzymatically active forms. While there were no significant catalytic differences among the forms, ORD and thermal stability experiments indicated that the electrophoretic forms differed in their conformations (4). Bovine heart cytochrome c components exhibit differences in light absorption, ORD, and CD spectra, as well as differences in oxidation-reduction potentials; these have been interpreted as indicating that the environment of the heme group,

specifically, the heme-protein interaction, has been affected, and supports a view that certain asparagine and glutamine residues are important in determining the conformation and biological activity characteristic of native mammalian cytochrome c (5).

A very powerful tool available today for the detection of protein heterogeneity is isoelectric focusing (IEF). Isoelectric focusing (or electrofocusing, isoelectric fractionation, isoelectric separation, stationary electrolysis, and isoelectric analysis), a relatively recently-applied form of electrophoresis, had its beginning many years ago. Isoelectric fractionation by electrical transport has been used for over 60 years, especially for group separations of acids and peptides. In 1912, Ikeda and Suzuki (6) obtained a patent for a "method of making a nutritive and flavoring substance." The substance was sodium glutamate, and it was produced by hydrolyzing vegetable proteins and electrolyzing the amino acid mixture in a three-compartment cell. The glutamic acid collected in the anode compartment. It was also noted that the basic amino acids could be isolated from the cathode compartment and the neutral ones from the center section. The resolving power of the method, however, stayed far behind that obtainable by moving boundary or zone electrophoresis, which used differences in electrophoretic mobility at a constant pH. A renewed interest in isoelectric methods was evoked by a series of experiments by Kolin (7-11), by Hoch and Barr

(12), and by MacDonald and Williamson (13). It was not until the theoretical foundations were laid by Svennson (14,15), that the technique became practical for routine analytical and preparative use.

Isoelectric focusing is the name most commonly used for the phenomenon that occurs when ampholytes are subjected to a potential difference in aqueous solution. Every ampholyte has an isoelectric point, its pI, the pH at which the net charge is zero. When a mixture of low molecular weight ampholytes is exposed to a potential gradient in a convection-free water solution, a pH gradient is formed, since each ampholyte will migrate to its isoelectric point and establish a pH where it is focused corresponding to its pI. Such a gradient, if protected from thermal and other disturbances, is very stable, since each ampholyte is in acid-base equilibrium at its pI. When a sample of proteins with isoelectric points within the pH range of the gradient is added to the gradient, the protein molecules will assume different charges determined by the pI of each protein and the pH where the protein is located. This results in each protein molecule migrating toward that pH in the system where it is isoelectric. The proteins are thus exactly focused at the point where the pH is equal to the pI.

The resolving power of this technique is quite impressive; the separation of proteins with pI differences of 0.02 pH units has been achieved (16). With the commercial availability of the carrier ampholytes needed to

establish the pH gradient, IEF has become a powerful technique for the separation of complex protein mixtures.

It has long been known in the hemoglobin field that spectra are sensitive to conformational, amino acid, or species differences, and there have been attempts to correlate spectral peak positions with changes in function, primary structure, and phylogenetic differences. In deoxyhemoglobins and myoglobins, the spectrum is invariant over the pH range in which the proteins are stable and is characterized by a single broad, asymmetrical band in the visible region with a maximum at about 555 nm. The Soret band in the near UV shows a maximum at 430 nm in hemoglobin and 435 nm in myoglobin. An interesting feature of the deoxyhemoglobin spectrum is its sensitivity to a number of modifications of the protein associated with abnormal functional behavior, in particular, with the loss of heme-heme interaction or cooperativity. For isolated α and β chains, for example, the extinction coefficient of the maximum in the Soret is 15% lower than that of the intact tetramer, and the maximum is shifted a few nm towards lower wavelengths (17). This type of spectrum was recognized as being characteristic of the quickly-reacting form of hemoglobin obtainable by flash photolysis of carbon monoxide hemoglobin (18). Hemoglobin H (β_4) (19), isolated α and β chains of human hemoglobin (17), hemoglobin digested by carboxypeptidase A (20), and the hemoglobin-haptoglobin complex (21) exhibit the same spectrum in the deoxygenated

form. These proteins all have much higher affinity for ligands than normal hemoglobin and do not show any heme-heme interaction. The altered spectrum and abnormal functional behavior seem to be associated with the absence of the characteristic ligand-linked change in conformation that is observed by X-ray analysis in normal hemoglobin. Like all the other ferrous liganded derivatives, oxyhemoglobin and oxymyoglobin show two absorption bands in the visible; the oxy forms have maxima at 577 nm and 541 nm for mammalian hemoglobins, and 581 nm and 542 nm for myoglobins In the overwhelming majority of oxyhemoglobins and myoglobins, the α peak (longest wavelength peak) has a larger extinction coefficient than the β peak. The Soret band lies near 415 nm. The visible spectrum is independent of pH in that region where the proteins are stable. Small but definite differences exist in the spectra of the oxygenated derivatives of hemoglobins from different species, which have been identified as the expression of species differences in the same protein (22). The difference between the maximum of the α band of the CO and the O_2 derivatives (known as the "span") varies in the different hemoglobins and myoglobins and has been correlated (23) with the relative affinity of any given protein for oxygen and carbon monoxide. The proposed relationship between the span in angstroms and the partition coefficient (M) between oxygen and carbon monoxide is given by

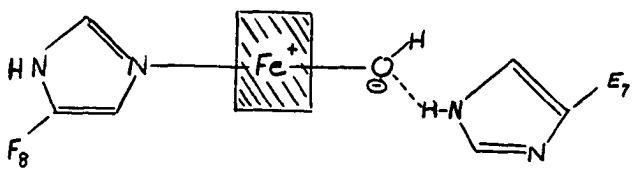
$$y = \log M/\text{span}$$

The ratio was found to lie in the range 0.045 to 0.050, and to be constant for several vertebrate hemoglobins and myoglobins. It has been proposed that such a linear correlation is to be expected on the basis of charge-transfer theory and thus provides evidence that binding of O and CO involves a charge-transfer process (24). However, this relation breaks down for invertebrate hemoglobins, such as Gastrophylus larvae hemoglobin (25) and Aplysia myoglobin (26).

It has been definitely established by the X-ray crystal structure determination of sperm whale ferrimyoglobin that the sixth coordination position of the ferric ion is occupied by a water molecule or by a hydroxyl ion (27). This coordinated water molecule is generally considered to dissociate according to the scheme

$$Fe^{3+}\text{——}H_2O \rightleftharpoons Fe^{3+}\text{——}OH^- + H^+$$

It has been proposed, however, that the heme linked ionization in ferric hemoglobin and myoglobin is due to the liberation of a proton from the distal (E7) imidazole, the iron ligand being always an OH^- (28).



The spectrum of ferric hemoglobin or myoglobin is pH sensitive. For acid ferrihemoglobins, the spectrum shows two bands in the visible with maxima at about 505 and 635 nm; the Soret band lies at about 405 nm. For myoglobins, the maxima are shifted towards longer wavelengths (20).

One of the more important functions to study in heme proteins is the ligand equilibrium behavior. Binding studies are valuable in that they can yield information about protein structure and the forces stabilizing such structures. If the initial binding of a ligand to a protein facilitates further ligand binding (cooperativity), binding is said to have destabilized the native conformation of the macromolecule. On the other hand, initial binding may result in stabilization of the native structure against conformational changes induced by other agents. The organization of the folded protein is therefore affected by ligand binding (29).

This phenomenon has been very clearly demonstrated in heme proteins. While the most widely studied equilibrium behavior of hemoglobin and myoglobin is the binding of oxygen, ferric interactions are of great interest also. Almost all of the investigations have been on the reactions of ferrihemoglobin with such ligands as azide, cyanide, thiocyanate, fluoride, hydroxide, hydrogen sulfide, and imidazole. For several of these ligands, such as hydroxide and fluoride, there is general agreement that the

equilibrium curve conforms to a simple formulation of the mass law with a single equilibrium constant. In the case of other ligands, particularly azide, there are conflicting reports, with some suggestion that there is substantial heme-heme interaction in ferrihemoglobin for this equilibrium (30). The most frequently reported behavior, however is that of a lack of cooperativity in both ferrihemoglobin and ferrimyoglobin (20).

The kinetics of the reactions of hemoglobins and myoglobins with ligands are equally as or even more important than the equilibrium studies in providing information on the structure-function relationships and on the mechanisms of reaction. Whereas oxygen equilibrium studies can demonstrate the phenomenon of heme-heme interaction, kinetic studies have been able to prove the intrinsic difference in reactivity towards oxygen between α chains and β chains of intact human ferrihemoglobin as well as a random sequence of binding for CO (31). Flash-photolysis studies of the rate of association of carbon monoxide and hemoglobin (18) and stopped-flow measurements of the rate of dissociation of oxygen from oxyhemoglobin (32,33) revealed the presence of a quickly-reacting form of hemoglobin, information unobtainable from other methods of investigation. Kinetic studies have shown clearly the nonequivalence of the α and β chains of intact human hemoglobin in the association rate constant for azide (34). In many situations,

therefore, kinetic studies can be very detailed and sensitive probes of the environment of the hemes.

Since most reactions of hemoglobins and myoglobins with ligands are rapid (half-time less than 1 sec), they require the use of special methods for measuring fast reactions in solution. A brief description of the two methods used in the studies reported here seems appropriate.

The first special method for the study of rapid reactions in solution was introduced in chemistry when Hartridge and Roughton measured the kinetics of the reaction of ferrohemoglobin with oxygen (35). The rapid-mixing methods they introduced have been the main experimental tool for the study of heme kinetics for 50 years. The variation most widely used at present is the stopped-flow method, which evolved from the original continuous-flow technique with the availability of rapid recording devices. Two solutions containing reagents, after being forced at high speed into a mixing chamber, enter into an observation tube which is located as close as possible to the mixing point. The flow is suddenly stopped by an appropriate device and the changes which occur in the portion of fluid present in the observation tube are followed with a rapid detecting system. The various types of stopped-flow apparatus which have been developed differ from one another especially in the way pressure is exerted on the solutions during flow, in the design of the mixing chamber and the observation tube, and in the stopping device (36,37). These technical

features acquire significance because they affect the "dead time" of the instrument, the time elapsed between initiation of mixing and the first useful observation. The apparatus that is routinely used in many laboratories is that of Gibson (36,38), which is commercially available.

All of the liganded derivatives of ferrous hemoglobins and myoglobins are photodissociable, although the quantum yield varies greatly from one ligand to another (39). This provides the basis for the flash-photolysis methods. When ligand-bound ferrous hemoglobin or myoglobin is exposed to the intense light pulse of an electronic flash, dissociation of the ligand occurs:

$$FeX \xrightarrow{h\nu} Fe + X$$

After the flash, the system returns to the equilibrium in the dark, and hence the recombination reaction can be followed with an appropriate monitoring system. The flash photolysis devices used in work with heme proteins follow the general features of that described by Porter (40). The high quantum yield for the dissociation of the carbon monoxide derivative, the one most often used in such studies, permits the use of relatively low energy flashes, and even commercial flash lamps used for photographic work may be successfully employed. The dead time of the flash photolysis apparatus depends on the characteristics of the light pulse and on the efficiency of the screening of the

stray light from the flash (41). Overall dead times from a few microseconds to tenths of milliseconds can be obtained. Flow-flash methods have been successfully employed in work on hemoglobin reactions (42).

If there is to be an attempt to interpret kinetic studies in structural-functional terms, then it is necessary to investigate a number of hemoglobins from widely varying sources in order to obtain a general understanding of heme protein kinetics and the extent to which the protein can influence heme behavior. By studying hemoglobins and myoglobins from animals far removed phylogenetically from humans, the best chances of looking at large differences in protein sequence are encountered. That these differences in primary structure can lead to dramatic differences in functional behavior can be seen in the hemoglobin from an echinoderm, Thyonella gemmata, where the CO association rate constant is the slowest yet reported for any hemoglobin, about 1/10,000th that for human hemoglobin H, and yet the oxygen association rate constant is really quite similar to that for human hemoglobin (43). Such studies provide unique opportunities to correlate structure and function in heme proteins.

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