Communication

Stoichiometry of Iron Binding by Uteroferrin and Its Relationship to Phosphate Content*

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The analysis of uteroferrin's iron content by an acid-release method used in previous studies shows a critical dependence on phosphate, i.e. the native phosphate-free enzyme yields two irons/molecule, while enzyme with one tightly bound phosphate gives closer to one iron. In contrast, two irons/molecule of protein are found in samples assayed by a wet ash method. When iron assays are carried out on samples of purple two-iron protein reductively stripped of their phosphate, both methods again yield two iron atoms/molecule. However, the discrepancy between the two methods recurs when phosphate is added to samples of pink protein which were formerly free of phosphate. These results suggest that phosphate bound to native uteroferrin may have interfered with iron determinations in some earlier studies. Furthermore, enzyme samples with one tightly bound phosphate have the optical purity index (i.e. $A_{550}/A_{455} \approx 14.0$) and extinction coefficient at 280 nm, characteristic of putative one-iron preparations. There is little doubt, therefore, that previous EPR, magnetic susceptibility, and iron titration experiments thought to have been carried out on genuine one-iron preparations were in fact done on samples of two-iron protein bearing a single tightly bound phosphate. Results of earlier studies indicate that the properties of putative one-iron preparations may be reconciled with those of the two-iron-phosphate-laden protein studied here.

Uteroferrin, an iron-binding, single chain, basic glycoprotein with phosphatase activity and a molecular weight between 35,000 and 40,000 (1-4) is isolatable from the uterine fluids of pregnant sows or pseudopregnant hormone-treated sows (5, 6). The protein can exist in either of two interconvertible forms, i.e. purple (oxidized), which is enzymatically inactive and EPR-silent (2-4, 6-9), or pink (reduced), which is enzymatically active and exhibits an intense rhombic EPR signal with $g_{ss} \approx 1.74$ (2-4, 6-9). Oxidation with peroxide or ferricyanide produces the purple form ($\lambda_{\text{max}} \approx 550$-570 nm) of the protein while mild reduction yields its pink form ($\lambda_{\text{max}} \approx 510$ nm) (4).

The iron-binding stoichiometry of uteroferrin has long been a source of sharp disagreement with claims of either one or two irons bound per molecule (1-4, 6-14). Since essentially the same preparative and analytic procedures have been used by all groups studying the protein, it is unlikely that such procedures are the source of this discrepancy (4). Disparities in molecular weight and extinction coefficients used to estimate protein concentration also exist, but these fail to account for the discrepancies in iron content and merely add to the perplexities of the iron-binding problem (2-4, 6, 10).

Herein, we report the results of iron and phosphate assays and dry weight determinations performed on samples of uteroferrin that were either pink or purple as isolated and bear up to one phosphate/molecule. These studies suggest that both pink and purple uteroferrin, isolated as described in the literature (2, 3, 6, 15, 16), naturally bear close to two irons/molecule. However, phosphate, which may be present in samples of the purple form of the protein, interferes with iron assays by acid-release methods, so that only about half the iron is detected by such assays.

MATERIALS AND METHODS

Preparation of Uteroferrin—Uterine fluid extracted from progesterone-treated sows is typically either pink with $\lambda_{\text{max}} \approx 510$-525 nm or purple with $\lambda_{\text{max}} \approx 545$ nm (4). When processed according to published procedures (4, 15, 16), the pink fluid yields the pink (reduced) form of uteroferrin, a form largely free of phosphate (Table I), while the purple fluid gives the purple (oxidized) form of the protein. The latter bears close to one phosphate/molecule (Table I). To minimize contamination by extrinsic metal ions, especially iron, the precautions described in Ref. 8 were taken. The optical purity index ($A_{550}/A_{455}$) of all freshly prepared protein samples was less than 15.0 and such samples were shown to be homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions (17). Samples were concentrated and reduced as previously described (8).

Iron Analyses—Triplicate iron analyses were carried out by the wet ash method of Cameron (18) except that Ferrazine (Hach Chemical Co., Ames, Iowa) and iron-free 1.0 M sodium acetate (pH 8.4) were substituted for o-phenanthroline and pyridine, respectively. Iron analyses were also done by a previously described colorimetric method using glacial acetic acid to affect iron release (7, 8). Iron contents of Fe$^{2+}$ and Fe$^{3+}$ solutions used in titration studies were determined as described in Ref. 8. As in the earlier study, no significant differences between measured and anticipated iron concentrations were found.

Phosphate Analyses—Assay for free inorganic and total phosphate were done as described in Ref. 19 with sample buffers serving as blanks.

Preparation of Samples Used in EPR and Optical Titration Studies—Protein samples and solutions of ferrous and ferric iron ions used in titration studies were prepared according to Ref. 8.

Determination of Extinction Coefficient at 280 nm—The dry weight of a measured volume of purple uteroferrin ($\lambda_{\text{max}} \approx 545$ nm) with an optical purity index of 13.5 was determined in triplicate in a manner similar to that described in Ref. 10, except that samples were heated to a constant weight in Pyrex vials in a vacuum oven at 106 °C for 6 h. The extinction coefficient was calculated from this weight and the absorbance at 280 nm.

EPR—EPR spectra were recorded according to procedures of Antanaitis et al. (9), taking the necessary precautions to prevent saturation of the Cu(II)-EDTA standard at 10 K.

Enzyme Activity—Phosphatase activity was determined as described by Kenough et al. (20). Samples that were purple as isolated ($\lambda_{\text{max}} \approx 545$ nm) gave low but variable turnover numbers ranging from 15 to 60 s$^{-1}$. Addition of a 5-fold excess of H$_2$O$_2$ to such samples abolished all activity. Pink protein shown to be free of phosphate had turnover numbers in the range of 450-460 s$^{-1}$, in agreement with previously published values (9, 15). In contrast, purple two-iron protein bearing one phosphate/molecule, but rendered pink by reduction, gave lower turnover numbers in the range of 200-250 s$^{-1}$, close to those reported for preparations of one-iron uteroferrin (2, 6).
Effect of Phosphate on Iron Assays of Uteroferrin

TABLE I

Relation between uteroferrin’s iron and phosphate contents as determined by acid-release and wet ash methods

| Sample                      | \( \lambda_{\text{max}} \) | \( \lambda_{\text{ase}}/\lambda_{\text{max}} \) | Iron by acid release (g atom/ mol) | Iron by wet ash (g atom/ mol) | Bound phosphate (mol/mol) |
|-----------------------------|-----------------------------|-----------------------------------------------|----------------------------------|----------------------------|--------------------------|
| Pink (as isolated)          | 512                         | 14.8                                          | 1.81                             | 1.97                       | 0.07                     |
| Purple (as isolated)        | 545                         | 13.6                                          | 1.19                             | 1.89                       | 0.78                     |
| One-iron (supplied by R. M. Roberts) | 545                     | 13.9                                          | 0.99                             | 1.46                       | 0.66                     |
| Purple 2Fe                  | 550                         | 16.2                                          | 1.63                             | 1.85                       | 0.21                     |
| (stripped of phosphate)     |                             |                                               |                                  |                            |                          |
| Pink 2Fe                    | 545                         | 13.8^d                                        | 1.19                             | 1.92                       | 0.81                     |
| (plus phosphate)            | (510)^a                     | (14.5)                                        | (1.79)                           | (1.83)                     | (0.06)                   |

*Total iron determined as described under “Materials and Methods” and protein concentrations estimated using \( \epsilon_{560} = 50 \text{ mM}^{-1} \text{ cm}^{-1} \) (Ref. 9) for low phosphate samples and 43.8 mM\(^{-1}\) cm\(^{-1}\) for high phosphate samples. The latter value is based on optical and dry weight data reported herein (Table II) and assumes a molecular weight of 35,000 (10).
*Total phosphate determined as described under “Materials and Methods.”
*Passage through a G-75 superfine column to remove denatured protein lowered this ratio to 15.0.
*This represents value after passage through a G-75 superfine column.
*Quantities in parentheses represent values for the pink protein prior to the addition of phosphate.

RESULTS AND DISCUSSION

As Table I shows, porcine uteroferrin may be isolated in two readily distinguishable forms, i.e. pink, characterized by \( \lambda_{\text{max}} = 510-520 \text{ nm} \) and an optical purity index above 14.0, or purple, with \( \lambda_{\text{max}} = 545 \text{ nm} \) and a purity index below 14.0. The iron content of such samples determined by the acid-release method depends critically on the protein’s phosphate content, with pink essentially phosphate-free enzyme yielding two irons/molecule and the purple protein with its complement of one tightly bound phosphate giving closer to one iron. In contrast, the wet ash method carried out on aliquots of the same samples consistently gives two irons/molecule regardless of the protein’s phosphate content (Table I). These results suggest that tightly bound phosphate may have confounded earlier assays based on acid-release methods (7, 8), causing purple two-iron phosphate-laden protein preparations to be mischaracterized as one-iron preparations (7, 8, 21).

To test this idea further, iron and phosphate analyses were repeated on a sample of purple protein that had been reductively stripped of its phosphate (20). As Table I shows, iron assays by the two methods are now in substantial agreement. Moreover, the small discrepancy in iron content between the two methods is apparently accounted for by the residual bound phosphate. In fact, the “missing iron” in all samples assayed correlates well with the sample’s phosphate content with the sum of acid-releasable iron and bound phosphate nearly equalling the protein’s iron content determined by the wet ash method. Similar assays of differic transferrin in the presence and absence of phosphate, in amounts equimolar with protein-bound iron, indicate that free phosphate itself has no effect on iron determinations in this protein.1 Taken together, these results suggest that it is iron associated with protein-bound phosphate that may elude detection by the acid-release method. In a complementary experiment, phosphate added to pink enzyme, formerly phosphate-free, caused the discrepancy in iron content to reappear (Table I).

Together these observations strongly suggest that previous EPR (7, 8), magnetic susceptibility (7), and iron titration experiments (8) thought to have been carried out on genuine one-iron preparations of uteroferrin were, in fact, done on samples of two-iron protein bearing a single tightly bound phosphate. In this regard, it should be noted that phosphate binding not only accounts for the low iron content of “one-iron preparations” but can also explain the puzzling discrepancy between the optical purity indices of two-iron and putative one-iron preparations (cf. Refs. 10 and 3). As revealed in Tables I and II, the purity indices of purple protein samples with close to one bound phosphate are uniformly lower than those without phosphate. Such low values coincide with those reported for one-iron uteroferrin (4, 6, 10). Furthermore, the decrease in the optical purity index of phosphate-laden protein samples is accompanied by a corresponding decrease in the protein’s extinction coefficient at 280 nm determined by dry weight methods (Table II), bringing it into the range recently reported for the so-called one-iron protein (10). As reported here, the small but not insignificant discrepancy in extinction coefficients between Roberts’ one-iron preparation and two-iron phosphate-laden protein most likely reflects minor differences in either the iron or phosphate content of the samples studied.

Properties of putative one-iron preparations may, by and large, be reconciled with those of the two-iron phosphate-laden protein. More specifically, if the concentration and iron content of the sample of putative one-iron protein used in susceptibility studies is corrected by assuming that the preparation actually contains two irons/molecule and that the appropriate value of \( \epsilon_{560} = 4000 \text{ M}^{-1} \text{ cm}^{-1} \) (3) rather than 3100 M\(^{-1}\) cm\(^{-1}\) (10), then the corrected effective magnetic moment \( \mu_{\text{eff}} \) becomes 1.25 Bohr magnetons/iron (7). This value falls very close to the 1.1 Bohr magnetons found for peroxide-oxidized \( \lambda_{\text{max}} \geq 550 \text{ nm} \) two-iron protein (12). The slightly higher value of \( \mu_{\text{eff}} \) for the so-called one-iron preparation may reflect a small difference in either the oxidation state or extraneous iron content of the two samples. As already reported (4, 9) strong saturation of the copper standard used to quantitate uteroferrin’s \( g = 1.74 \) EPR signal in two previous studies (7, 8) led to overestimation of the purple protein’s unpaired spin concentration. Reassessment of EPR data gathered for the purple protein indicates that no more than 10% of its iron may be accounted for by the \( g = 1.74 \) signal (9, 14). Thus, earlier quantitative EPR and susceptibility data of putative one-iron preparations of uteroferrin are in accord with the corresponding data for the two-iron phosphate-laden protein. Furthermore, \( \text{Fe}^{2+} \) and \( \text{Fe}^{3+} \) titration studies repeated on samples of two-iron phosphate-laden protein (results not shown) yield essentially the same results obtained in earlier

1 B. C. Antamaitis, unpublished observations.
studies with so-called one-iron protein, i.e. incremental addition of ferrous iron to purple uteroferrin steadily increases the intensity of its $g^* = 1.74$ EPR signal until it reaches a level 3.3 times the original. The saturation level is achieved when the added iron corresponds to 200% of the iron originally present in the sample. Addition of ferrous iron also shifts the protein's visible absorption maximum from 545 to 525 nm with no significant change in its intensity. Furthermore, addition of Fe$^{3+}$ to pink (reduced) uteroferrin shifts its absorption maximum to 525 nm giving a sample whose EPR signal intensity matches that of its ferrous iron-produced counterpart. The greater increase in signal intensity found in the present titration study most likely reflects a weaker signal in the purple protein used here. We have observed that the intensity of the weak signal detected in purple samples not oxidized by peroxide (8) varies by as much as a factor of two, even in samples with $\lambda_{\text{max}} = 545$ nm. It appears, therefore, that results of the previous titration study on so-called one-iron uteroferrin may be explained by assuming that Fe$^{3+}$ and Fe$^{2+}$, respectively, reduce and oxidize one of the two atoms in purple and pink uteroferrin (4, 9). Such redox changes are in accord with the currently accepted spin-coupled binuclear iron model for the protein's active site (4, 9, 11–14). In the earlier of these titration studies, it was also reported that the intensity of the pink protein's signal was little changed from that of the purple protein (8). In that study, however, the sample of pink protein to which no iron was added was optically assayed immediately after its passage through the G-25 column ($\lambda_{\text{max}} = 515$ nm) while the EPR signal was quantitated after the sample had set overnight in the cold room. A similarly prepared two-iron phosphate-containing sample, when reduced and passed through the same column, retained =15% of its original phosphate and, left overnight in the cold room, largely reoxidized producing a sample with $\lambda_{\text{max}} \sim 540$ nm. Such samples with EPR signal intensity only 15–20% greater than the original purple protein's signal led to the erroneous conclusion that the intensity of the pink protein's signal is not significantly greater than that of the untreated purple protein (7, 8).

Binding of phosphate may explain another puzzling aspect of uteroferrin's behavior, i.e. the small but reproducible variation observed in the position of the oxidized protein's absorption maximum (4). When oxidized with peroxide, the absorption maximum of freshly prepared phosphate-free samples shifts to 565–570 nm (4, 9, 11, 22). In contrast, the absorption maximum of phosphate- (or molybdate-) laden protein shifts only to 550 nm, in a manner reminiscent of so-called one-iron preparations of uteroferrin (4, 6, 9, 11).

As reported here, phosphate binding to uteroferrin interferes with iron assays based on acid-release methods. Yet, according to earlier studies, such iron assays of the spleen protein, which also binds one phosphate/molecule, do not suffer the same fate (23). The reason for this is not known. It may, however, be related to the fact that the bovine splenic enzyme, unlike uteroferrin, consists (at least as isolated) of two fragments which are likely to separate under the strongly acidic and reducing conditions of the iron assay, rendering both of that protein's ions more accessible to the Fe (II) chromogens used in such assays (3, 4, 24, 25).

Buhl et al. (10) have reported that reductive acid-release methods and atomic absorption spectroscopy alike give close to one-iron atom in their preparations. As Table I shows, iron and phosphate assays of a "one-iron" preparation, kindly supplied by R. M. Roberts (University of Florida at Gainesville), do yield somewhat lower iron values than those found for other samples tested; nonetheless, the acid-release and wet-ash assays exhibit a sizeable difference in iron content, a difference that is again strongly correlated with the sample's phosphate content. Perhaps phosphate binding introduces a systematic error in iron assays by atomic absorption spectroscopy as well. In our own studies, we have noted that undigested samples of iron-bearing proteins give variables results when assayed by atomic absorption spectroscopy with a graphite furnace.

All in all, these comparative studies of two-iron phosphate-free protein, two-iron phosphate-laden protein, and putative one-iron protein lead to the conclusion that uteroferrin is isolated only as a two-iron protein which, depending upon its history, may have up to one tightly bound phosphate/molecule. What is actually found in a given preparation is signaled by the color of the uterine fluid from which it is purified, so that pink fluid yields protein with little or no phosphate, and purple fluid ($\lambda_{\text{max}} = 545$ nm) gives protein with close to one tightly bound phosphate.

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| Sample | $\lambda_{\text{max}}$ | $\lambda_{\text{max}}/\lambda_{\text{max}}$ | $d_{280}$ | Bound phosphate | Ref. |
|--------|----------------|-----------------|--------|---------------|-----|
| Pink (as isolated) | 515 | 14.7 | 14.3 | 0.06 | 9 |
| Purple (as isolated) | 545 | 13.6 | 12.5 | 0.78 | This work |
| Purple (as isolated) | 545 | 13.2–14.0 | 11.5 | Not given | 10 |
| Pink-purple (mixture) | 530 | 15.2 | 14.3 | Not given | 3 |

* Extinction coefficient at 280 nm of a 1% protein solution based on optical and dry weight data from sources indicated.

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